IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit: 1644
KALTOFT, et al.	)	Examiner: SAUNDERS, D.
Serial No.: 09/720,371	)	Washington, D.C.
Filed: April 30, 2001	)	
For: METHODS OF EXPANDING AND SELECTED DISEASE ASSOCIATED T-CELLS	)	Docket No.: KALTOFT=1
	)	Confirmation No.: 2534

DECLARATION OF KELD KALTOFT

U.S. Patent and Trademark Office
Customer Service Window
Randolf Building
401 Dulany Street
Alexandria, VA 22314

Sir:

I, Keld Kaltoft, hereby declare:

1. I am the first-named inventor of the above-identified application, and I am knowledgeable in the fields of cell biology and immunlogy. My curriculum vitae is enclosed.
2. I have reviewed the office action mailed March 4, 2005, and the prior art cited against the application.
3. All three of the prior art references (Flyer, Riddel, Habermann) teach the use of peripheral blood mononuclear cells (PBMC) as feeder cells in cultivating T-cells. There are always natural killer cells in PBMC preparations; these are about 5-15 % of the PBMC cells (Mandler RN et al.: Beta-endomorphin augments the cytolytic activity and interferon production of natural killer cells. J.Immunol (1986) 136; 934-39).
4. Natural killer cells would be expected to eliminate co-cultivated disease associated antigen-activated continuous cytotoxic T cell lines, and hence it would not be expected that the latter cells, even if stimulated by cytokines such as IL-2 and IL-4, would exceed the Hayflick limit of about 23 +/-7 PD (population doublings). In particular, they would not be expected to reach the presently claimed levels of at least 40, 50, 60, 100, 150 or 200 PD.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Keld Kaltoft

31/8 -05

Keld Kaltoft Date

A A R H U S U N I V E R S I T E T

Curriculum vitae Keld Kaltoft

1978: Master degree of Science in Chemistry

1978-88: Assistant Professor in Genetics, University of Aarhus, Denmark

1988- : Associate Professor in Genetics, University of Aarhus Denmark

2000: Founder of the company CellCure

Has been an author or co-author on 63 peer reviewed articles with emphasis on T lymphocyte biology in cancer and chronic inflammatory diseases

Ectopic Human Telomerase Catalytic Subunit Expression Maintains Telomere Length But Is Not Sufficient for CD8$^+$ T Lymphocyte Immortalization[1]

Marco Migliaccio,*§ Mario Amacker,† Tom Just,‡ Patrick Reichenbach,† Danila Valmori,* Jean-Charles Cerottini,* Pedro Romero,* and Markus Nabholz[2†]

Like most somatic human cells, T lymphocytes have a limited replicative life span. This phenomenon, called senescence, presents a serious barrier to clinical applications that require large numbers of Ag-specific T cells such as adoptive transfer therapy. Ectopic expression of hTERT, the human catalytic subunit of the enzyme telomerase, permits fibroblasts and endothelial cells to avoid senescence and to become immortal. In an attempt to immortalize normal human CD8$^+$ T lymphocytes, we infected bulk cultures or clones of these cells with a retrovirus transducing an hTERT cDNA clone. More than 90% of transduced cells expressed the transgene, and the cell populations contained high levels of telomerase activity. Measuring the content of total telomere repeats in individual cells (by flowFISH) we found that ectopic hTERT expression reversed the gradual loss of telomeric DNA observed in control populations during long term culture. Telomere length in transduced cells reached the levels observed in freshly isolated normal CD8$^+$ lymphocytes. Nevertheless, all hTERT-transduced populations stopped to divide at the same time as nontransduced or vector-transduced control cells. When kept in IL-2 the arrested cells remained alive. Our results indicate that hTERT may be required but is not sufficient to immortalize human T lymphocytes. ***The Journal of Immunology,*** **2000, 165: 4978–4984.**

Like most normal human cells, T lymphocytes have a limited life span. In vitro proliferation of human T cells, widely used for research and clinical purposes, depends on the continuous presence of IL-2 as well as on periodic stimulation with Ag, mitogens, or a combination of agonistic Abs such as anti-CD3 and anti-CD28. In these conditions, T lymphocytes undergo a finite number of cell divisions until a certain point where they stop proliferating. This state has been called replicative senescence by analogy with the term used to designate the growth arrest of diploid human fibroblasts after a finite number of cell divisions (reviewed in Ref. 1). Senescent T lymphocytes fail to proliferate in response to renewed attempts at stimulation, which frequently lead to massive cell death by apoptosis (2), but they can be maintained in culture for a long time, usually several months, if fresh IL-2 is added periodically. Senescent T cells are able to up-regulate IL-2Rα surface expression in response to Ag stimulation (3). Expression of other surface markers is generally unchanged, with a few exceptions, notably the decline in levels of the costimulatory receptor CD28 (4–6). Senescent CD4$^+$ T cells are still able to secrete a number of cytokines when stimulated (5), and senescent CD8$^+$ T cells retain high levels of Ag-specific cytotoxicity (3).

The impossibility to propagate T lymphocytes indefinitely limits their use for clinical applications that require large cell numbers such as adoptive transfer therapy of infections and malignancies. Why cultured T cells stop proliferating at a certain point remains unclear. In fibroblasts, telomere shortening during cell division is the molecular clock that triggers the entry of cells into senescence (7, 8). Expression of the enzyme telomerase allows germline cells and tumor cells to maintain telomere length during proliferation. Ectopic expression of human telomerase catalytic subunit (hTERT)[3] is sufficient to permit some cell types, such as fibroblasts, retinal pigment epithelial cells, and endothelial cells (7, 8), to avoid senescence and to proliferate indefinitely. These immortalized cells continue to display normal cellular functions and do not undergo changes characteristic of malignant transformation (9, 10). Other cell types require alterations in cell cycle regulatory elements, besides telomerase expression, for immortalization. In particular, blocking of the Rb/p16 pathway together with telomerase expression are required to immortalize human keratinocytes and breast epithelial cells (11).

Telomerase expression can be detected in hemopoietic stem cells, where it is transiently up-regulated during in vitro cytokine-driven expansion (12, 13). Telomerase activity is also detectable in different stages of T lymphocyte differentiation (14). In vitro, mature T cells transiently express telomerase activity in response to stimulation with specific Ag (15), mitogens, or anti-CD3/anti-CD28 Abs (14, 16, 17). However, if the same cultures are subjected to repeated cycles of stimulation, the peak of telomerase activity becomes progressively lower (16). The ability of lymphocytes to express telomerase activity is exceptional among normal differentiated human cells. It has been proposed that up-regulation

*Division of Clinical Oncoimmunology, Ludwig Institute for Cancer Research, University Hospital, Lausanne, Switzerland; †Swiss Institute for Cancer Research (ISREC), Epalinges, Switzerland; ‡Department of Probe Applications, Dako A/S, Glostrup, Denmark; and §Department of Biochemistry, Public University of Navarra, Pamplona, Spain

Received for publication March 14, 2000. Accepted for publication August 8, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by grants from the Swiss National Science Foundation and Krebsforschung Schweiz (to M.N.). M.A. was supported by a Swiss Cancer League Grant to J. Lingner.

[2] Address correspondence and reprint requests to Dr. Markus Nabholz, Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland. E-mail address: markus.nabholz@isrec.unil.ch

[3] Abbreviations used in this paper: hTERT, human telomerase catalytic subunit; hTER, human telomerase RNA template subunit; CFSE, 5-carboxyfluorescein diacetate-succinimidyl ester; MSCV, mouse stem cell virus; TRAP, telomerase repeat amplification protocol.

0022-1767/00/$02.00

of telomerase allows T lymphocytes to preserve their replicative potential during clonal expansion so that memory cells with a strong capacity for expansion can be generated (18). However, a negative correlation between telomere length and donor age has been observed in $CD4^+$ T lymphocytes, and peripheral blood T lymphocytes with a memory phenotype have shorter telomeres than naive cells (19, 20). Telomeres also shorten in in vitro cultured lymphocytes (19, 20), and both telomere length and CD28 expression decline with the number of cell divisions (5, 21). To investigate whether ectopic hTERT expression leads to immortalization of normal human CTL we infected bulk cultures or clones of human $CD8^+$ lymphocytes with a retrovirus transducing an hTERT cDNA clone. We report that although the transduced cells express high levels of telomerase and maintain telomere length, they do not have a significantly longer life span.

Materials and Methods

Purification and culture of $CD8^+$ T lymphocytes

PBMC were obtained from healthy donors by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and $CD8^+$ cells were purified by magnetic cell sorting using a miniMACS device (Miltenyi Biotec, Auburn, CA). The $CD8^+$ CTL clones NH 55 and LAU 203 0.3/3 were obtained by limiting dilution culture in the presence of PHA, irradiated allogeneic feeder cells, and IL-2 as previously described (22). Clone NH 55 was obtained from peptide-stimulated PBMC of normal donor NM (23) and clone LAU 203 0.3/3 from tumor-infiltrating lymph node cells of melanoma patient LAU 203 (24).

Purified fresh $CD8^+$ T cells and $CD8^+$ CTL clones were plated at 10^5 cells/well in Iscove's medium (Life Technologies, Basel, Switzerland) supplemented with 10% human serum, asparagine, arginine, and glutamine in the presence of 150 U/ml recombinant human IL-2 (a gift from Glaxo, Geneva, Switzerland) and stimulated with 1 μg/ml PHA (Life Technologies) plus irradiated allogeneic PBMC (3000 rad) as feeder cells, as described elsewhere (22). In the case of freshly isolated $CD8^+$ T cells, additional cycles of stimulation were performed at intervals of 2 or 3 wk. Population doublings were determined by weekly counts of viable cells. To estimate the mean number of cell divisions vs time, we stained cell samples with the diacetate form of the carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) once or twice per week, and analyzed them by flow cytometry 6 h, as well as 4 and 7 days after staining (25, 26). A senescent population of clone NH 55 was stained at the same times and used as a standard. Living cells were gated and peaks corresponding to cells that had undergone a definite number of divisions were identified in the fluorescence histograms. From a histogram with N peaks,

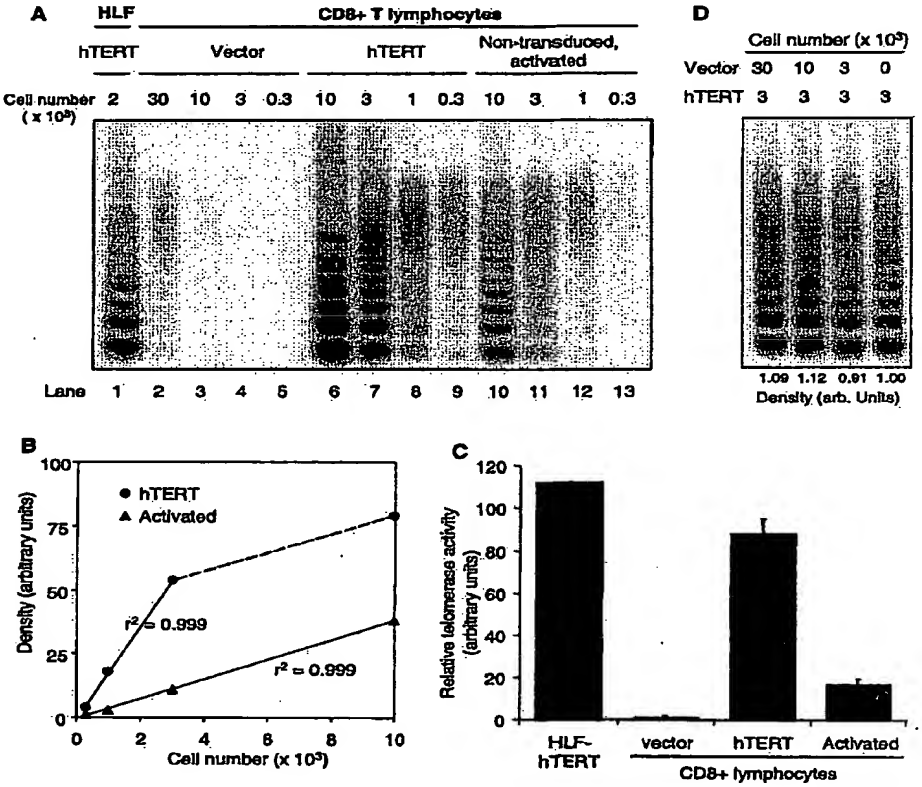

FIGURE 1. Telomerase activity in $CD8^+$ lymphocytes transduced with empty vector or hTERT, or stimulated with anti-CD3/anti-CD28 Abs. *A*, Nuclear extracts from cells transduced with hTERT (*lanes 6–9*) or the empty vector (*lanes 2–5*) were prepared 3 wk after the initial stimulation, when the transiently induced endogenous telomerase activity declined to low levels. To compare maximal endogenous telomerase activity with that due to the transduced hTERT construct, we also stimulated fresh cells from the same donor with a combination of anti-CD3 and anti-CD28 Abs and extracted cells 5 days later (*lanes 10–13*). Extracts corresponding to the indicated cell number were assayed by TRAP. Extracts from hTERT-transduced human lung fibroblasts (HLF) were used as control (*lane 1*). For each lane, the relative telomerase activity was determined by measuring with a phosphor imager the intensity of the repeat bands. *B*, Linear correlation between the amount of extract and telomerase activity in hTERT-transduced lymphocytes and activated cells at the peak of endogenous telomerase expression. Note that the signal given by 10^4 hTERT-transduced cells is out of the linear range of the assay and was not used for quantification. *C*, Mean activities and SD of the different cell populations were plotted and compared with the activity in cells transduced with the vector, which was arbitrarily set to 1. *D*, The reaction shown in *lane 6* was repeated in the presence of increasing amounts of extracts from lymphocytes transduced with the empty vector. No significant differences were observed, indicating the absence of inhibitors in the telomerase-negative cells.

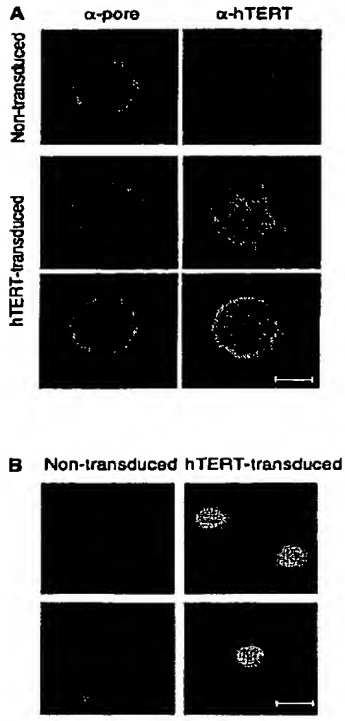

FIGURE 2. Ectopic expression of hTERT at the single-cell level. Indirect immunofluorescence of fixed $CD8^+$ T lymphocytes. *A*, Double-staining with anti-nuclear pore Ab (*left*) and anti-hTERT Ab (*right*) of nontransduced (control) and hTERT-transduced cells from bulk cultures. Bar, 5 μm. *B*, Staining with anti-hTERT Ab of nontransduced cells (control; *left*) and hTERT-transduced cells (*right*) of the CTL clone LAU 203 0.3/3. Bar, 15 μm. Note that the pictures in (*A*) were acquired on a confocal microscope, whereas (*B*) shows pictures taken with a lower resolution light microscope.

the mean number of cell divisions (MNCD) was calculated according to the formula

$$\text{MNCD} = \sum_{i=1}^{N} n_i X_i$$

where n_i is the number of cell divisions corresponding to peak i and X_i is the fraction of cells in this peak.

To induce endogenous telomerase activity, complete PBMC (10^6 cells/ml) were stimulated with immobilized anti-CD3 (OKT3, 10 μg/ml) plus soluble anti-CD28 (CK248, 1 μg/ml) in the presence of 150 U/ml of IL-2. After 5 days of culture, $CD8^+$ cells were isolated by magnetic sorting, and telomerase activity was determined.

Retrovirus construction and infection

The hTERT retroviral construct was made by PCR amplification of full-length hTERT cDNA that was inserted into the mouse stem cell virus (MSCV) pac vector (27) provided by Dr. D. R. Littman (New York University Medical Center, New York, NY). Amphotropic viruses were generated by the transfection of this plasmid into ΦNX cells (provided by Dr. G. Nolan, Stanford University, Stanford, CA) as described (28). $CD8^+$ bulk cultures and clones were infected 3 days after stimulation according to a spin infection protocol (29) repeated three times at daily intervals. The efficiency of transduction, estimated by staining with specific anti-hTERT Ab (see below), varied from 1 to 5%. Transduced cells were selected in 1.0 μg/ml puromycin for 1 wk.

Telomerase repeat amplification protocol (TRAP) assays and immunofluorescence

Nuclear extracts were prepared as described (30) and TRAP was performed according to (31) using the ACX-anchored return primer. Relative signal intensity of the repeat bands was measured by phosphor imaging analysis. To compare telomerase activity of the different cell populations, the intensity of the signals from individual reactions was normalized to the cell equivalents of extract added. Signals that fell into the range in which there was a linear correlation between signal intensity and cell equivalents were used for quantification (see Fig. 1*C*). For indirect immunofluorescence, cells were diluted in complete culture medium, dropped onto glass slides coated with polylysine, and incubated for 2 h at 37°C. After washing and fixation with 4% paraformaldehyde in PBS for 15 min at room temperature, cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min before blocking with 2% BSA/0.1% Tween 20 in PBS for 30 min at room temperature. After washing, cells were incubated with primary Abs for 1 h at room temperature. Mouse anti-nuclear pore Ab (32) was a gift from Dr. Susan Gasser (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). Rabbit anti-hTERT Ab (0.4 μg/ml) was affinity-purified and will be described elsewhere (M. Amacker and J. Lingner, manuscript in preparation). Incubation with secondary Abs, Alexa 488-conjugated goat anti-rabbit Ab (Molecular Probes), and Cy3-conjugated goat anti-mouse Ab (The Jackson Laboratory, Bar Harbor, ME) were conducted for 45 min at room temperature. Images were acquired on a Zeiss LSM 410 confocal microscope (Fig. 2*A*) and on a Coolview Photonics CCD Zeiss Axiophot microscope (Fig. 2*B*).

Telomere length determination by flowFISH

To measure cellular telomere length, cells were hybridized in situ with a fluorescent telomere-specific peptide nucleic acid probe, according to previously described, slightly modified protocols (20, 33, 34). To follow the evolution of a single population of lymphocytes during long-term culture, cell samples were frozen at different time points and analyzed in a single flowFISH experiment. Frozen cells (10^5-5×10^5) were thawed, washed in PBS, and resuspended to 10^5 cells/100 μl of a hybridization mixture (Dako, Glostrup, Denmark) containing 70% dimethylformamide and a telomere-specific FITC-conjugated $(C_3TA_2)_3$ peptide nucleic acid probe. After 10 min at 82°C samples were incubated overnight at room temperature in the dark. Control samples were resuspended in hybridization solution without probe to obtain background fluorescence values. After hybridization, cells were spun down and washed twice with 4 ml PBS at 40°C for 10 min and finally resuspended in PBS containing 0.1% BSA, 10 μg/ml RNase A (Boheringer Mannheim, Indianapolis, IN), and 0.1 μg/ml propidium iodide (Calbiochem-Novabiochem, La Jolla, CA). After 4 h at room temperature in the dark, cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) or stored at 4°C before analysis. Signals from FITC-labeled beads (Quantum premixed; Flow Cytometry Standards, San Juan, PR) were measured at the beginning and at the end of every experiment. Signals were acquired in linear scale mode, and events were gated according to propidium iodide fluorescence to restrict analysis to cells with diploid DNA content, as described before (33).

Results

Expression of telomerase activity in hTERT-transduced $CD8^+$ T lymphocytes

We isolated $CD8^+$ T cells from peripheral blood of healthy donors by magnetic sorting and stimulated them with irradiated allogeneic PBMC and PHA to trigger cell proliferation. Three days later, the cell population was infected with an amphotropic MSCV virus (27) containing the puromycin *N*-acetyl transferase gene (conferring resistance to puromycin) under the control of the phosphoglycerol kinase promoter and a complete hTERT cDNA under the control of the viral long terminal repeat. As expected from the work of others (7, 8), primary human lung fibroblasts infected with the same virus expressed telomerase activity and did not undergo senescence (data not shown).

Infected lymphocytes were selected in puromycin, and telomerase activity was measured in nuclear extracts of resistant cells by TRAP. As shown in Fig. 1, *A* and *C*, transduced lymphocytes expressed high levels of telomerase activity, similar to those found in fibroblasts transduced with the same construct. Enzyme activity was ~80 times higher than that in cells derived from the same

lymphocyte population but transduced with a control vector. These results indicate that the introduced hTERT cDNA is expressed in T lymphocytes as efficiently as in fibroblasts, and that in both cell types hTERT is the component that limits telomerase activity. Nuclear extracts from cells transduced with the empty vector or the hTERT construct were prepared 3 wk after stimulation with PHA, when the transiently induced endogenous telomerase activity had declined to barely detectable levels. For any given extract there was a linear correlation between the signals due to telomerase products (as measured by phosphor imager) and the amount of extract added (Fig. 1*B*), indicating that the efficiency of the PCR step did not vary between reactions. Addition of increasing amounts of extract from vector-transduced, telomerase-negative cells to a TRAP reaction with 3000 cell equivalents of hTERT-transduced lymphocytes did not significantly affect the level of telomerase activity measured (Fig. 1*D*), indicating that at the concentrations used, lymphocyte extracts do not inhibit telomerase activity.

To compare maximal endogenous telomerase activity with the one due to the transgene, we also performed TRAP assays with extracts of $CD8^+$ T cells activated with immobilized anti-CD3 Ab plus soluble anti-CD28 Ab, a method that has been reported to efficiently induce telomerase activity in cultured human T cells (14, 17). Extracts were prepared 5 days after stimulation, at the peak of induced telomerase activity. As shown in Fig. 1, the activity detected in activated normal lymphocytes was approximately four times lower than that in hTERT-transduced cells. This difference could reflect low endogenous enzyme activity in all activated normal cells, or high telomerase levels restricted to a small fraction of cells. To address this question we stained cells, by indirect immunofluorescence, with an Ab specific for hTERT (M.Amacker and J. Lingner, manuscript in preparation). Observation by confocal microscopy showed a pattern of distinct spots in the nucleus, as previously described for tumor cells (35), in >90% of the lymphocytes transduced with hTERT, whereas only background fluorescence was detected in nontransduced control cells (Fig. 2*A*). No strongly stained cells were detectable among lymphocytes activated with anti-CD3/anti-CD28 Abs (data not shown), indicating that the difference between the average telomerase activity of the transduced and activated control lymphocytes is mainly due to a lower level of endogenous hTERT in all normal activated cells. Transduced cells could be expanded for several weeks with no loss of hTERT expression.

In addition to bulk cultures of freshly isolated $CD8^+$ T lymphocytes we infected two Ag-specific CTL clones with the same retroviral hTERT construct. Clone NH 55 recognizes influenza matrix (FluMA) peptide 58–66 (23), whereas clone LAU 203 0.3/3 is specific for the tumor-associated Ag Melan-A_{26-35} (24) (see *Materials and Methods*). Light microscopy of cells stained with anti-hTERT Abs revealed that >90% of hTERT-transduced cells expressed high levels of hTERT protein, whereas control cells did not show any staining above background (Fig. 2*B*). At the resolution obtained with the light microscope the punctuate pattern seen in Fig. 2*A* is not resolved.

Ectopic hTERT expression does not prevent growth arrest of $CD8^+$ T lymphocytes

To determine whether ectopic hTERT expression extended the lifespan of T lymphocytes, we expanded cells infected with MSCV virus containing hTERT cDNA or with the empty vector (carrying only the puromycin resistance gene), as well as nontransduced cells, by periodic stimulation with PHA plus irradiated PBMC in the presence of IL-2. Living cells were counted at least once a week to determine the number of population doublings. Bulk cultures of nontransduced $CD8^+$ T lymphocytes underwent 16–19 population doublings before proliferation ceased, in agreement with previous reports (36, 37). Cell populations expressing the hTERT transgene stopped growing after a similar number of population doublings as matched control populations (Fig. 3, *A* and *B*). After growth arrest, cells not only failed to proliferate when restimulated with PHA and irradiated feeder cells, but massive cell death by apoptosis was often observed instead (data not shown).

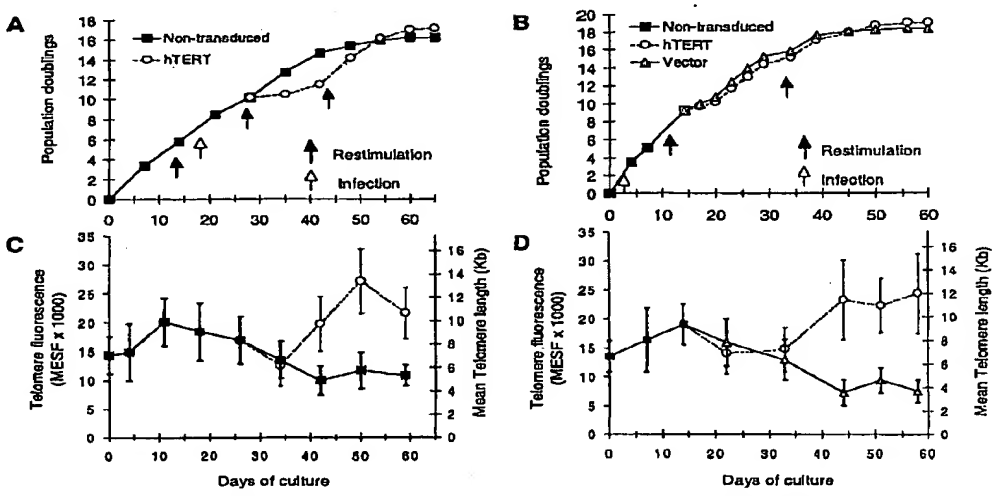

FIGURE 3. In vitro growth and telomere length evolution of $CD8^+$ human T lymphocytes. Cells were cultured and stimulated as described in *Materials and Methods*. Infections with hTERT constructs or empty vectors were conducted on days 16–19 (experiment 1) or on days 4–6 (experiment 2). Population doublings were determined by counting viable cells once or twice per week (*A* and *B*). We assume that puromycin-resistant cells undergo the same number of population doublings during selection as the nontransduced control population. Telomere fluorescence was measured by flowFISH (see Fig. 5). Mean fluorescence signals were plotted (*C* and *D*) as molecular equivalents of soluble fluorochrome (MESF) after subtraction of the background (left *y*-axis scale) and transformed into telomere length estimates (20) (right *y*-axis scale). Error bars represent the SD from the mean of the fluorescence histograms.

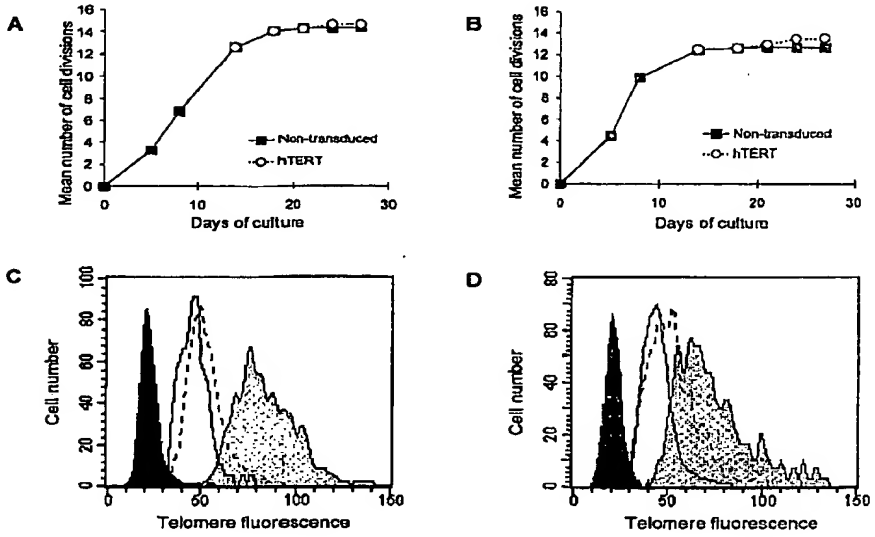

FIGURE 4. In vitro growth and telomere length evolution in CTL clones NH 55 and LAU 203 0.3/3. Infections with hTERT retroviral constructs were conducted on days 4–6. Mean numbers of cell division were determined by weekly staining of cells with CFSE and analysis of CFSE fluorescence histograms 3 and 7 days later (*A* and *B*). Telomere length was measured by flowFISH (*C* and *D*). *Black*, Background; *no shading/dashed line*, cells at the time of transduction (day 4); *no shading/solid line*, nontransduced control cells on day 30; *gray*, hTERT-transduced cells on day 30.

The apparent lag in the growth kinetics of the transduced population shown in Fig. 3*A* probably reflects the effect of inhibitory products generated by dying cells eliminated by the puromycin selection. Note that no lag is observed when growth curves of cells transduced with the hTERT construct and with the empty virus are confronted (Fig. 3*B*).

Similar experiments were performed with two Ag-specific CTL clones, whose rate of proliferation was continuously followed by CFSE staining (25, 26). At weekly intervals, cell aliquots were stained with CFSE and analyzed by flow cytometry 3 and 7 days later to estimate the mean number of cell divisions during these periods. Comparison of the CFSE data with viable cell counts indicated that cells underwent about twice as many divisions as population doublings. Similarly to what we had observed in the bulk cultures, hTERT transduction did not affect the proliferative capacity of either CTL clone (Fig. 4, *A* and *B*). CFSE staining of cells after growth arrest revealed that the cells had ceased to divide. This indicates that the constant number of living cells in the arrested cultures is not the consequence of equilibrium between cell division and cell death.

Ectopically expressed hTERT maintains CD8$^+$ T lymphocyte telomeres

We used flowFISH to monitor telomere length in cultured CD8$^+$ T lymphocytes. The technique quantifies, by flow cytometry, the fluorescent signals from individual cells after in situ hybridization with a fluorescent peptide nucleic acid probe specific for telomere repeats (20, 33, 34). Frozen aliquots of cells collected at different times from the same population were all processed in the same experiment. Cells were stained with propidium iodide, and telomere fluorescence was measured only in G1 phase cells. Representative histograms are shown in Fig. 5. The fluorescence of every individual cell is proportional to the total telomere length of its chromosomes. An estimate of the mean telomere length is obtained by comparing cellular signals with those from fluorescent bead standards, applying the equation derived by Rufer et al. (20).

An increase in telomere fluorescence of freshly isolated CD8$^+$ T lymphocytes could be observed during the first 2 wk of culture after the initial stimulation. Thereafter telomere signals gradually declined until the time when the cells stopped dividing (Fig. 3, *C* and *D*). In contrast, transduction with hTERT cDNA resulted in a progressive increase in telomere length. When the cells stopped proliferating, telomeres had reached an average length slightly higher than the peak observed 2 wk after the first stimulation. The coefficient of variation of all the fluorescence histograms is similar, indicating that the decrease in telomere length during culture as well as the increase after hTERT transduction affects the entire population of cells and not only a subpopulation. This homogeneity correlates well with the uniform staining of the transduced cell

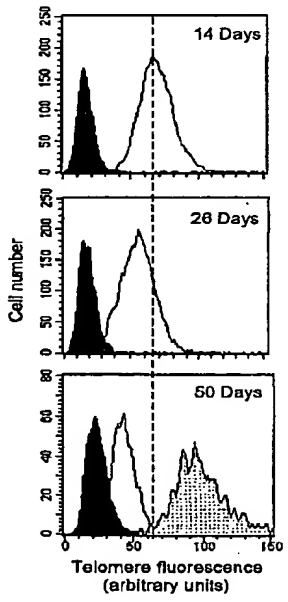

FIGURE 5. Typical examples of telomere fluorescence histograms. Total telomere fluorescence in single cells of CD8$^+$ T lymphocyte bulk cultures was measured by flowFISH. *Black*, background; *no shading*, control cells (transduced with empty vector); *gray*, hTERT-transduced cells.

populations with anti-hTERT Abs. Telomere fluorescence measurements in transduced and nontransduced CTL clones after 30 days of culture show that ectopic hTERT expression results in telomere lengthening also in these cells (Fig. 4, *C* and *D*).

Discussion

Most differentiated human cells do not express telomerase activity, and their telomeres shorten during proliferation. This can be prevented by ectopic expression of hTERT. Freshly isolated, mature T lymphocytes are peculiar in the sense that they transiently express telomerase activity upon activation; in this respect they resemble hemopoietic stem cells in expansion (13). However, the amplitude of telomerase expression declines with repeated stimulation cycles, and telomeres do shorten (16). We have found that telomere length increases in freshly isolated T cells during 2 wk after the first stimulation, before it starts to decline. This suggests that during the initial period following T lymphocyte activation, endogenous telomerase activity is sufficient to increase telomere length. However, it might also reflect selection for naive T cells with long telomeres. Telomere lengthening has also been described in normal, in vivo proliferating B lymphocytes (19). Results from a recent study (38) suggest that both hTERT and the RNA template subunit of telomerase (hTER) are up-regulated after lymphocyte activation, and that there is a correlation between the levels of hTER expression and telomerase activity. Our experiments with hTERT-transduced T lymphocytes cultured for extended periods show that hTER levels are not limiting.

As in fibroblasts (7), ectopic expression of hTERT induces telomere lengthening in T cells. But unlike fibroblasts or endothelial cells (39), hTERT-transduced T lymphocytes cease to proliferate after a similar number of population doublings or cell divisions as control cells. This indicates that telomere shortening is not the only process that limits the life span of T cells. However, it cannot be excluded that one or a few telomeres continue to shorten in hTERT-transduced cells and trigger growth arrest when they reach a critical size. To rule out this unlikely hypothesis it will be necessary to quantify individual telomere length by Q-FISH (40).

Our results are reminiscent of the finding that immortalization of human keratinocytes and breast epithelial cells depends on additional changes besides hTERT expression, namely, on inactivation of the Rb protein/p16 pathway (11). One explanation for the different requirements for the immortalization of fibroblasts, on the one hand, and certain epithelial cells, on the other, is that the culture conditions for the latter are suboptimal and lead to activation of the p16/Rb stress pathway. A similar explanation may apply to T lymphocytes. Progressive p16 accumulation during T cell in vitro proliferation has indeed been reported (41), but the culture system used did not allow restimulation of cells with PHA, suggesting that it was inadequate for maximal expansion of T cells by this method.

An alternative explanation for the growth arrest of $CD8^+$ T lymphocytes under the conditions used in our experiments may be that the stimulation requirements of T lymphocytes change during clonal expansion as part of a differentiation process of naive into memory cells. Thus, the apparent senescence of these cells may not be due to the intrinsic limitation of the proliferative capacity of the cells, but rather to the loss of the ability to proliferate in response to the stimuli provided (PHA + feeder cells plus IL-2). One change that might reduce responsiveness is the decrease in the expression of the costimulatory receptor CD28 in T cells that have undergone a high number of divisions. It has been reported that CD28-mediated costimulation is necessary to prevent cell death during T cell activation (42) and that CD28 expression declines during in vitro culture and is virtually absent in senescent cells (43). We have compared CD28 expression on lymphocytes transduced with hTERT or with the control vector, at different times of culture and in senescent cells (data not shown). In both populations we observed a similar gradual loss of CD28 expression during culture. Loss of CD28 may be one of the causes why growth-arrested T cells respond to restimulation with PHA and feeder cells by apoptosis rather than proliferation. Down-regulation of other costimulatory molecules such as CD134 (OX-40) and CD154 (CD40L), or IL receptors such as IL-4R, IL-7R, and IL-15R and the IL-2R common γ chain (4) may also affect responsiveness of these cells to TCR-mediated signaling. According to this hypothesis, transduction of $CD8^+$ T cells with components that restore their capacity to respond to the same signals as naive cells should allow the cells to proliferate further until their telomeres become critically short. We predict that, as in fibroblasts, the latter limit can indeed be overcome by ectopic expression of hTERT. Thus, hTERT may be required but is not sufficient to immortalize human T lymphocytes.

Ackowledgements

We thank Renate Milesi for her excellent technical assistance and Natalie Rufer for her advice concerning the flowFISH method. Glaxo (Geneva, Switzerland) provided a generous supply of recombinant IL-2. We are grateful to Susan Gasser for anti-nuclear pore Abs, to Gary Nolan for ΦNX cells, and to Don Littman for the MSCV plasmid.

References

1. Effros, R. B., and G. Pawelec. 1997. Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion? *Immunol. Today 18:450.*
2. Grubeck-Loebenstein, B., H. Lechner, and K. Trieb. 1994. Long-term in vitro growth of human T cell clones: can postmitotic "senescent" cell populations be defined? *Int. Arch. Allergy Immunol. 104:232.*
3. Perillo, N. L., F. Naeim, R. L. Walford, and R. B. Effros. 1993. The in vitro senescence of human T lymphocytes: failure to divide is not associated with a loss of cytolytic activity or memory T cell phenotype. *Mech. Ageing Dev. 67:173.*
4. Pawelec, G., A. Rehbein, K. Haehnel, A. Merl, and M. Adibzadeh. 1997. Human T-cell clones in long-term culture as a model of immunosenescence. *Immunol. Rev. 160:31.*
5. Adibzadeh, M., H. Pohla, A. Rehbein, and G. Pawelec. 1995. Long-term culture of monoclonal human T lymphocytes: models for immunosenescence? *Mech. Ageing Dev. 83:171.*
6. Nociari, M. M., W. Telford, and C. Russo. 1999. Postthymic development of $CD28^-CD8^+$ T cell subset: age-associated expansion and shift from memory to naive phenotype. *J. Immunol. 162:3327.*
7. Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright. 1998. Extension of life-span by introduction of telomerase into normal human cells. *Science 279: 349.*
8. Vaziri, H., and S. Benchimol. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol. 8:279.*
9. Morales, C. P., S. E. Holt, M. Ouellette, K. J. Kaur, Y. Yan, K. S. Wilson, M. A. White, W. E. Wright, and J. W. Shay. 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat. Genet. 21:115.*
10. Jiang, X. R., G. Jimenez, E. Chang, M. Frolkis, B. Kusler, M. Sage, M. Beeche, A. G. Bodnar, G. M. Wahl, T. D. Tlsty, and C. P. Chiu. 1999. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat. Genet. 21:111.*
11. Kiyono, T., S. A. Foster, J. I. Koop, J. K. McDougall, D. A. Galloway, and A. J. Klingelhutz. 1998. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature 396:84.*
12. Hiyama, K., Y. Hirai, S. Kyoizumi, M. Akiyama, E. Hiyama, M. A. Piatyszek, J. W. Shay, S. Ishioka, and M. Yamakido. 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol. 155:3711.*
13. Engelhardt, M., R. Kumar, J. Albanell, R. Pettengell, W. Han, and M. A. Moore. 1997. Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. *Blood 90:182.*
14. Weng, N. P., B. L. Levine, C. H. June, and R. J. Hodes. 1996. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J. Exp. Med. 183:2471.*
15. Hathcock, K. S., N. P. Weng, R. Merica, M. K. Jenkins, and R. Hodes. 1998. Antigen-dependent regulation of telomerase activity in murine T cells. *J. Immunol. 160:5702.*
16. Weng, N. P., L. D. Palmer, B. L. Levine, H. C. Lane, C. H. June, and R. J. Hodes. 1997. Tales of tails: regulation of telomere length and telomerase activity during

lymphocyte development, differentiation, activation, and aging. *Immunol. Rev. 160:43.*

17. Liu, K., M. M. Schoonmaker, B. L. Levine, C. H. June, R. J. Hodes, and N. P. Weng. 1999. Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes. *Proc. Natl. Acad. Sci. USA 96: 5147.*
18. Maini, M. K., M. V. Soares, C. F. Zilch, A. N. Akbar, and P. C. Beverley. 1999. Virus-induced $CD8^+$ T cell clonal expansion is associated with telomerase up-regulation and telomere length preservation: a mechanism for rescue from replicative senescence. *J. Immunol. 162:4521.*
19. Weng, N. P., L. Granger, and R. J. Hodes. 1997. Telomere lengthening and telomerase activation during human B cell differentiation. *Proc. Natl. Acad. Sci. USA 94:10827.*
20. Rufer, N., W. Dragowska, G. Thornbury, E. Roosnek, and P. M. Lansdorp. 1998. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat. Biotechnol. 16:743.*
21. Monteiro, J., F. Batliwalla, H. Ostrer, and P. K. Gregersen. 1996. Shortened telomeres in clonally expanded $CD28^-CD8^+$ T cells imply a replicative history that is distinct from their $CD28^+CD8^+$ counterparts. *J. Immunol. 156:3587.*
22. Valmori, D., N. Gervois, D. Rimoldi, J. F. Fonteneau, A. Bonelo, D. Lienard, L. Rivoltini, F. Jotereau, J. C. Cerottini, and P. Romero. 1998. Diversity of the fine specificity displayed by HLA-A*0201-restricted CTL specific for the immunodominant Melan-A/MART-1 antigenic peptide. [Published erratum appears in *1999 J. Immunol. 163:1093.*] *J. Immunol. 161:6956.*
23. Bednarek, M. A., S. Y. Sauma, M. C. Gammon, G. Porter, S. Tamhankar, A. R. Williamson, and M. S. Zweerink. 1991. The minimum peptide epitope from the influenza virus matrix protein: extra and intracellular loading of HLA-A2. *J. Immunol. 147:4047.*
24. Romero, P., N. Gervois, J. Schneider, P. Escobar, D. Valmori, C. Pannetier, A. Steinle, T. Wolfel, D. Lienard, V. Brichard, et al. 1997. Cytolytic T lymphocyte recognition of the immunodominant HLA-A*0201-restricted Melan-A/MART-1 antigenic peptide in melanoma. *J. Immunol. 159:2366.*
25. Wells, A. D., H. Gudmundsdottir, and L. A. Turka. 1997. Following the fate of individual T cells throughout activation and clonal expansion: signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J. Clin. Invest. 100:3173.*
26. Hasbold, J., A. V. Gett, J. S. Rush, E. Deenick, D. Avery, J. Jun, and P. D. Hodgkin. 1999. Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester. *Immunol. Cell. Biol. 77:516.*
27. Hawley, R. G., F. H. Lieu, A. Z. Fong, and T. S. Hawley. 1994. Versatile retroviral vectors for potential use in gene therapy. *Gene Ther. 1:136.*
28. Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA 90:8392.*
29. Clay, T. M., M. C. Custer, J. Sachs, P. Hwu, S. A. Rosenberg, and M. I. Nishimura. 1999. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J. Immunol. 163:507.*
30. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. *Nucleic Acids Res. 17:6419.*
31. Kim, N. W., and F. Wu. 1997. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res. 25:2595.*
32. Gotta, M., T. Laroche, A. Formenton, L. Maillet, H. Scherthan, and S. M. Gasser. 1996. The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type *Saccharomyces cerevisiae*. *J. Cell Biol. 134:1349.*
33. Rufer, N., T. H. Brummendorf, S. Kolvraa, C. Bischoff, K. Christensen, L. Wadsworth, M. Schulzer, and P. M. Lansdorp. 1999. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J. Exp. Med. 190:157.*
34. Hultdin, M., E. Gronlund, K. Norrback, E. Eriksson-Lindstrom, T. Just, and G. Roos. 1998. Telomere analysis by fluorescence in situ hybridization and flow cytometry. *Nucleic Acids Res. 26:3651.*
35. Harrington, L., W. Zhou, T. McPhail, R. Oulton, D. S. Yeung, V. Mar, M. B. Bass, and M. O. Robinson. 1997. Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev. 11:3109.*
36. Perillo, N. L., F. Naeim, R. L. Walford, and R. B. Effros. 1993. In vitro cellular aging in T-lymphocyte cultures: analysis of DNA content and cell size. *Exp. Cell. Res. 207:131.*
37. Weng, N. P., B. L. Levine, C. H. June, and R. J. Hodes. 1995. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proc. Natl. Acad. Sci. USA 92:11091.*
38. Weng, N., B. L. Levine, C. H. June, and R. J. Hodes. 1997. Regulation of telomerase RNA template expression in human T lymphocyte development and activation. *J. Immunol. 158:3215.*
39. Yang, J., E. Chang, A. M. Cherry, C. D. Bangs, Y. Oei, A. Bodnar, A. Bronstein, C. P. Chiu, and G. S. Herron. 1999. Human endothelial cell life extension by telomerase expression. *J. Biol. Chem. 274:26141.*
40. Poon, S. S., U. M. Martens, R. K. Ward, and P. M. Lansdorp. 1999. Telomere length measurements using digital fluorescence microscopy. *Cytometry 36:267.*
41. Erickson, S., O. Sangfelt, M. Heyman, J. Castro, S. Einhorn, and D. Grander. 1998. Involvement of the Ink4 proteins p16 and p15 in T-lymphocyte senescence. *Oncogene 17:595.*
42. Noel, P. J., L. H. Boise, J. M. Green, and C. B. Thompson. 1996. CD28 costimulation prevents cell death during primary T cell activation. *J. Immunol. 157:636.*
43. Effros, R. B. 1997. Loss of CD28 expression on T lymphocytes: a marker of replicative senescence. *Dev. Comp. Immunol. 21:471.*

Original Paper

Experimental and Clinical Immunogenetics

Exp Clin Immunogenet 1998;15:84–89 Received: December 19, 1997

Keld Kaltoft

Institute of Human Genetics, The Bartholin Building, University of Aarhus, Aarhus, Denmark

Cytokine-Driven Immortalization of in vitro Activated Human T Lymphocytes

CD28 Expression Correlates Inversely with Cell Population Doublings

Key Words
T lymphocytes
Immortality
Telomerase
Cytokines
CD28

Abstract

Like other normal human somatic cells, T lymphocytes are believed to have a finite in vitro life span. However, continuous T lymphocyte cell lines can often be established from chronic inflammatory skin diseases when the culture medium is supplemented with IL-2 and IL-4 but without antigen and accessory cells added. Based on the assumption that these continuous T lymphocyte cell lines were activated by antigen during the chronic inflammation taking place in vivo, I investigated whether peripheral blood T lymphocytes could be induced to cytokine-dependent continuous growth following antigen activation. Upon allostimulation, peripheral blood CD4+ T lymphocytes reproducibly escape from cellular senescence. These IL-2- and IL-4-dependent continuous T cell lines show high telomerase activity. Withdrawal of either IL-2 or IL-4 results in cell growth arrest concomitant with down-regulation of telomerase activity. When cultured continuously, these CD4+ human T lymphocytes gradually lose expression of CD28.

Introduction

The concept of cellular senescence is based on the belief that human somatic cells in vitro are constrained by replicative senescence. This phenomenon has been well documented for human fibroblasts [1, 2], keratinocytes and hepatocytes [3, 4]. Human T lymphocytes from chronic inflammatory skin diseases, however, seem to be an exception to this dogma, as they often escape from replicative senescence when cultured in a medium sup-

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0254–9670/98/0152–0084$15.00/0

Accessible online at:
http://BioMedNet.com/karger

Keld Kaltoft
Institute of Human Genetics
The Bartholin Building, University of Aarhus
DK-8000 Aarhus C (Denmark)
Tel. +45 8942 1673, Fax +45 8612 3173, E-Mail kaltoft@humgen.aau.dk

plemented with IL-2 and IL-4 [5, 6]. As neither addition of antigen nor antigen-presenting cells were required to establish continuous growth, these T lymphocytes seemed to be activated in vivo in such a manner that continuous in vitro growth only required addition of a sufficient cytokine source. As a chronic inflammation is expected to be maintained by antigen activation of T lymphocytes, I wanted to test the possibility that antigen stimulation could lead to cytokine-dependent continuous growth of peripheral blood T cells.

Materials and Methods

Derivation of Finite and Continuous Peripheral Blood T Cell Lines

Peripheral blood mononuclear cells (PBMC) from 3 healthy donors were isolated by standard Ficoll-Isopaque gradient centrifugation. The PBMC were resuspended at 5×10^5 cells/ml in 90% RPMI-1640, 10% human AB serum, 1,000 U/ml IL-2 and 500 U/ml IL-4 with antibiotics as described [5]. To establish whether longevity of cultured PBMC is dependent on in vitro activation, PBMC were cultured in the above medium alone or with additional alloactivation. 5×10^6 PBMC were stimulated with the heavily γ-irradiated (60 Gy) Psor-2 cell line at a 5:1 ratio. The Psor-2 cell line is a continuous T cell line established from a skin biopsy specimen of a patient with psoriasis vulgaris by culturing the skin specimen in the medium mentioned above [6].

Estimation of CD28 Expression as a Function of Cell Population Doublings

Monoclonal antibodies against CD3, CD4, CD8, CD28, and CD56 were purchased from PharMingen. An α/β T cell receptor subfamily antibody against $V_\beta 18$ was obtained from Immunotech. An indirect immunofluorescence technique was applied to label the cells as previously described [5]. Allostimulated continuously growing peripheral blood T cell lines were cryopreserved for each 10 PD. Cells cryopreserved at different cell population doublings (PD) were then thawed, cultured for 4 days and analyzed for CD28 expression by flow cytometry. CD4 and CD8 expression served as positive and negative controls, respectively. For each antibody, 2×10^4 cells were analyzed (FACS Calibur, Becton Dickinson). Fluorescence microscopy was also applied to evaluate the stainings.

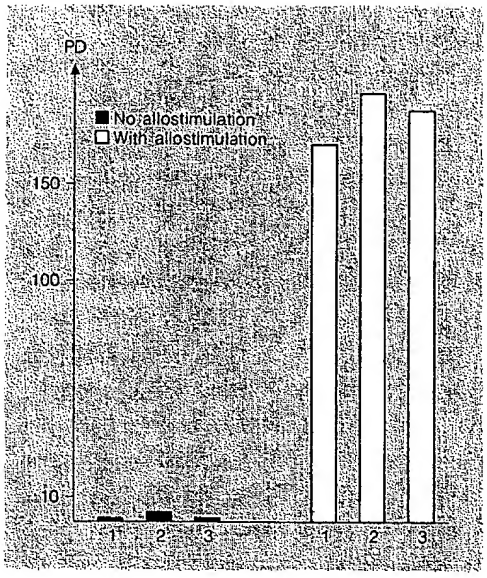

Fig. 1. Number of PD of three PBMC cultures grown in medium with IL-2 + IL-4 alone (left) or with allostimulation in the presence of IL-2 + IL-4 (right).

A clonal CD4+, $V_\beta 18$+ T cell line My-La, 46,XY,i(18q) [7, 8] cultured with 1,000 U/ml IL-2 and 500 U/ml IL-4 was also analyzed for CD28 expression at different PD.

Other Methods

Cells were found to be free of mycoplasma by the Hoechst staining test. Telomerase activity of 10^3 cells was determined by the TRAPeze Telomerase Detection Kit as described by the manufacturer (Oncor).

Results

Growth of PBMC with and without Allostimulation

PBMC from the 3 healthy donors proliferated between 1 and 3 PD when cultured in the cytokine-supplemented medium alone (fig. 1) in agreement with previously published data showing that PBMC proliferate only tran-

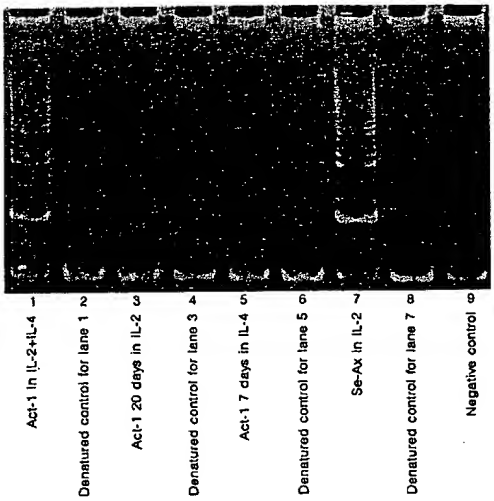

Fig. 2. Telomerase activity at 100 PD of a continuous peripheral-blood-activated CD4+ cell line (Act-1) cultured with IL-2 + IL-4, IL-2 or IL-4 as indicated. For comparison, telomerase activity of the leukemic cell line Se-Ax, cultured with IL-2 alone, is also shown.

siently when stimulated with a combination of IL-2 + IL-4 [5, 6]. However, when PBMC were allostimulated once with the Psor-2 cell line in the presence of a high concentration of IL-2 and IL-4, T cells as well as non-T cells (preferentially CD3–, CD56+) proliferated vigorously during the first 4–6 weeks.

After approximately 50 PD, only CD4+ T cell grew in the cytokine-based medium. All three CD4+ allostimulated T cell lines have proliferated beyond 150 PD with a PD time of 30–36 h (fig. 1). This corresponds to an increase in cell numbers of $2^{150} \approx 10^{45}$-fold. As allostimulated peripheral blood T lymphocytes have been estimated to have a limited in vitro lifespan of 23 ± 7 PD [9], the allostimulated CD4+ cell lines reported here can be considered continuous, effectively having an unlimited replication capacity.

So far, the three continuous peripheral-blood-derived CD4+ cell lines show no sign of growth exhaustion and at PD 150 still retain alloreactivity (results not shown).

Cytokine-Dependent Continuous T Cell Lines Have Cytokine-Dependent Telomerase Activity

Continuous cell lines are expected to have telomerase activity. When cultured in the presence of both IL-2 and IL-4, in-vitro-activated peripheral blood CD4+ T cells show high telomerase activity (fig. 2) comparable to that of a leukemic cell line Se-Ax [10], established from a patient with Sézary syndrome. Withdrawal of either IL-2 or IL-4 results in growth arrest. After withdrawal of IL-4, a 100 PD cell culture ceases proliferating after 14–21 days. Withdrawal of IL-2 results in cell growth arrest between 6 and 9 days. As shown in figure 2, telomerase activity in IL-2 or IL-4 starved cells is severely reduced. The results indicate that simultaneous presence of IL-2 and IL-4 regulates both growth and telomerase activity in these T cell lines.

CD28 Expression Correlates Inversely with Cell Population Doublings

Allostimulated PBMC cultured in the cytokine-supplemented medium became pure CD4+ cell lines after approximately 50–60 PD. CD28 expression of one such CD4+ cell line, Act-1, at PD 60 and PD 150 is presented in figure 3. CD28 expression is clearly detectable at PD 60 but absent at PD 150. A gradual decline in expression of CD28 between PD 60 and PD 150 could be observed (results not shown).

To investigate whether the culture system preferentially expands preexisting CD28-negative CD4+ cells or whether CD28 could serve as a mitotic clock in individual T cells, a clonal CD4+, V_β18+ T cell line established from an inflammatory skin biopsy specimen

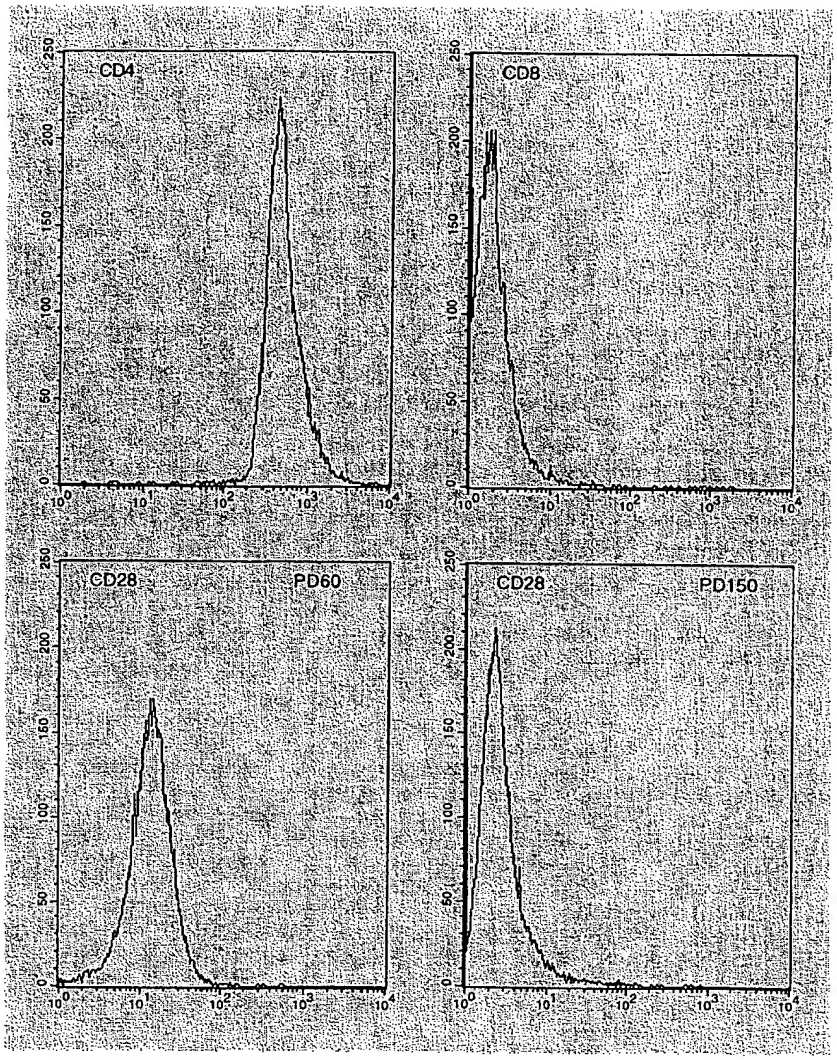

Fig. 3. CD28 expression of the continuous peripheral-blood-derived CD4+ cell line Act-1 at PD 60 and 150 compared with CD4 and CD8 expression at PD 150.

[7, 8] was investigated for CD28 expression. As shown in figure 4, CD28 expression of this T cell clone (My-La, 46,XY,i(18q)) decreases gradually with cell population doublings being present at PD 40 and completely absent at PD 200. However, CD4+ expression is compatible at PD 40 and PD 200. These findings are in agreement with data obtained from finite CD4+ T cell lines [11] showing downregulation of CD28, but not complete loss of

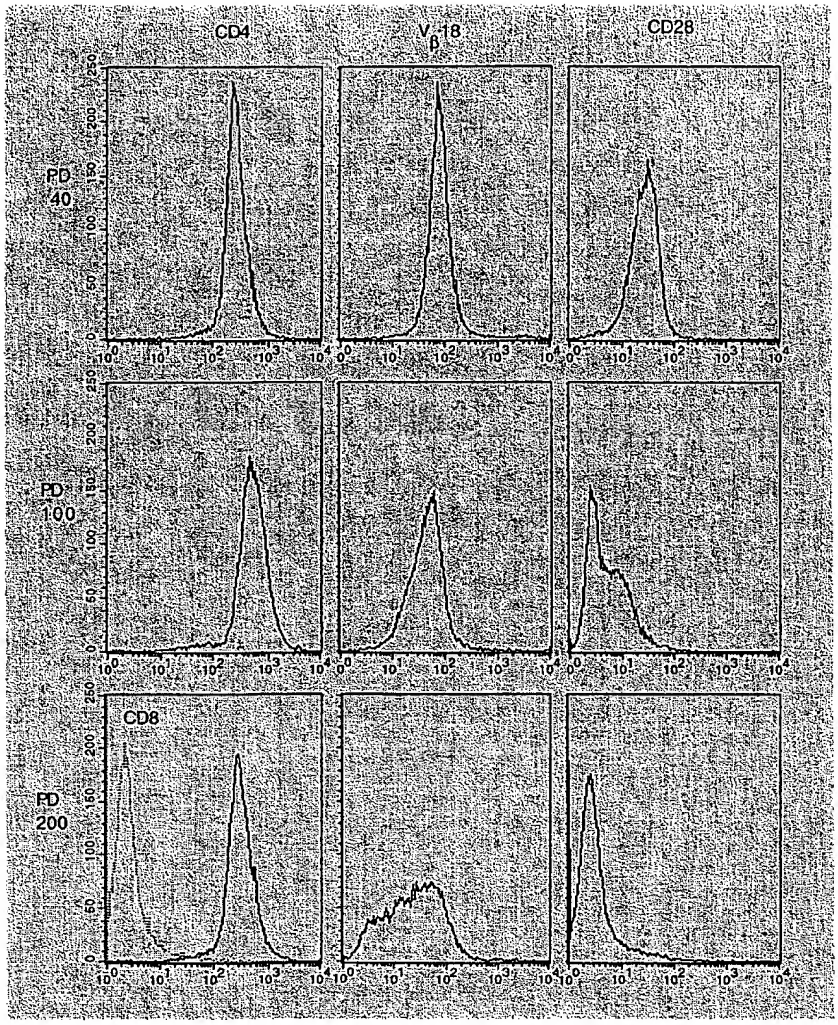

Fig. 4. CD28 expression at different PD of the clonal T cell line My-La, 46,XY,i(18q). Also shown is CD4 and $V_\beta 18$ expression at the different PD and CD8 expression at PD 200.

CD28 expression with increasing PD. The results presented here show that CD28 expression correlates inversely with cell population doublings and indicates that CD28 expression can serve as a mitotic clock at the clonal level.

Discussion

We have previously shown that continuous human T lymphocyte cell lines can often be established from chronic inflammatory skin diseases by addition to the culture medium of

only a cytokine source but without antigen and accessory cells added. As the interaction between dendritic cells and T lymphocytes in vivo seems to play an important role in the maintenance of chronic inflammatory skin diseases [12], it was hypothesized that activation of human T lymphocytes in vitro could also lead to establishment of cytokine-driven continuous growth. The results presented here show that alloactivation with the continuous psoriatic T cell line Psor-2 can efficiently prime allogeneic CD4+ peripheral blood T cells to cytokine-dependent continuous growth. These cytokine-driven peripheral-blood-derived CD4+ T cell lines show IL-2- and IL-4-dependent telomerase activity, and gradually lose CD28 expression with increasing PD.

In summary, contrary to other normal human somatic cells, T lymphocytes can be activated to continuous cytokine-driven growth in vitro like in vivo. The results presented here raise the possibility of generating an unlimited number of T cells with predefined specificity. Such immortal T cell lines may be useful for several applications, for instance for standardization of T-cell-mediated biological assays and for generating sufficient numbers of autoimmune T cells for human T cell vaccination.

Acknowledgements

I am grateful to Birgit Holm Hansen, Hanne Jacobsen, and Thrine Schneidermann for excellent technical assistance. I thank Schering-Plough Research Institute (Kenilworth, N.J., USA) for their generous gift of IL-4. This work was supported by a grant to Dansk Center for Molekylær Gerontologi.

References

1 Hayflick L, Moorhead PS: The serial cultivation of human diploid strains. Exp Cell Res 1961;25:585–621.
2 Goldstein S: Replicative senescence: The human fibroblast comes of age. Science 1990;249:1129–1133.
3 Rheinwald JG, Green H: Epidermal growth factor and multiplication of cultured human epidermal keratinocytes. Nature 1977;265:421–424.
4 Le Guilly Y, Simon M, Lenoir P, Bourel M: Long-term culture of human adult liver cells: Morphological changes related to in vitro senescence and effect of donor's age on growth potential. Gerontologia 1973;19:303–313.
5 Kaltoft K, Pedersen CB, Hansen BH, Lemonidis AS, Frydenberg J, Thestrup-Pedersen K: In vitro genetically aberrant T cell clones with continuous growth are associated with atopic dermatitis. Arch Dermatol 1994;287:42–47.
6 Kaltoft K, Hansen BH, Pedersen CB, Pedersen S, Thestrup-Pedersen K: Common clonal chromosome aberrations in cytokine-dependent continuous human T-lymphocyte cell lines. Cancer Genet Cytogenet 1995;85:68–71.
7 Kaltoft K, Bisballe B, Dyrberg T, Boel E, Rasmussen PB, Thestrup-Pedersen K: Establishment of two continuous T-cell strains from a single plaque of a patient with mycosis fungoides. In Vitro Cell Dev Biol 1992;28A:161–167.
8 Kaltoft K, Pedersen CB, Hansen BH, Thestrup-Pedersen K: Appearance of isochromosome 18q can be associated with in vitro immortalization of human T lymphocytes. Cancer Genet Cytogenet 1995;81:13–16.
9 Perillo NL, Walford RL, Newman MA, Effros RB: Human T lymphocytes possess a limited in vitro life span. Exp Gerontol 1989;24:177–187.
10 Kaltoft K, Bisballe S, Rasmussen HF, Thestrup-Pedersen K, Thomsen K, Sterry W: A continuous T-cell line from a patient with Sézary syndrome. Arch Dermatol Res 1987;279:293–298.
11 Effors RB, Pawelec G: Replicative senescence of T cells: Does the Hayflick limit lead to immune exhaustion? Immunol Today 1997;18:450–454.
12 Cooper KD: Atopic dermatitis: Recent trends in pathogenesis and therapy. J Invest Dermatol 1994;102:128–137.

Telomerase levels control the lifespan of human T lymphocytes

Alexander Röth, Hans Yssel, Jérôme Pène, Elizabeth A. Chavez, Mike Schertzer, Peter M. Lansdorp, Hergen Spits, and Rosalie M. Luiten

The loss of telomeric DNA with each cell division contributes to the limited replicative lifespan of human T lymphocytes. Although telomerase is transiently expressed in T lymphocytes upon activation, it is insufficient to confer immortality. We have previously shown that immortalization of human CD8$^+$ T lymphocytes can be achieved by ectopic expression of the human telomerase reverse transcriptase (hTERT) gene, which encodes for the catalytic component of the telomerase complex. To study the role of endogenous hTERT in the lifespan of human T cells, we blocked endogenous hTERT expression by ectopic expression of dominant-negative (DN) hTERT. Cells expressing DN-hTERT had a decreased lifespan and showed cytogenetic abnormalities, including chromosome ends without detectable telomeric DNA as well as chromosome fusions. These results indicate that while endogenous hTERT cannot prevent overall telomere shortening, it has a major influence on the longevity of human T cells. Furthermore, we show that up-regulation of hTERT in T cells upon activation decreases over time in culture. Long-term–cultured T cells also show a decreased expression of *c-myc* upon activation, resulting in less *c-myc*–induced transcription of hTERT. Moreover, memory T cells, which have expanded in vivo upon antigen encounter, expressed a lower level of hTERT upon activation than naive cells from the same donor. The observed inverse correlation between telomerase levels and replicative history suggests that telomerase levels in T cells are limiting and increasingly insufficient to sustain their proliferation. (Blood. 2003;102:849-857)

Introduction

Human T lymphocytes have a limited lifespan. During long-term culture, human T cells proliferate for a restricted number of cell divisions, after which the cells cease to proliferate and become senescent.[1] Therefore, in vitro–established antigen-specific CD8$^+$ or CD4$^+$ T clones can usually not be expanded beyond 20 to 30 population doublings,[2,3] and subcloning of established human T-cell clones usually fails or yields subclones with very little replicative potential. The proliferative capacity of T cells may therefore be linked to the replicative history of the cells. Replicative senescence of T cells has also been demonstrated in vivo: memory T cells have a decreased proliferative capacity and shorter telomeres compared with naive T cells,[4,5] and the telomere length in lymphocytes shows a progressive decrease with donor age.[4-7]

It has been suggested that the limited lifespan of most human somatic cells is due to progressive telomere shortening.[8,9] Telomeres shorten during each cell division until they reach a critical length, at which point the cells undergo cell-cycle arrest and enter the nondividing state known as replicative senescence. Replicative senescence can be overcome by expression of the enzyme complex telomerase, which consists of a catalytic subunit with reverse transcriptase activity, human telomerase reverse transcriptase (hTERT), a RNA template, human telomerase RNA component (hTERC), and associated proteins.[10] Ectopic expression of the hTERT gene in human cells has been shown to result in telomere elongation and immortalization of various cell types, including fibroblasts, endothelial cells, and retinal pigment epithelial cells.[11,12] We have previously reported that ectopic hTERT expression in CD8$^+$ T cells leads to an extended lifespan, which demonstrates that loss of telomere ends is a limiting factor for the lifespan of T cells.[13,14]

In contrast to most other somatic cell types, T cells express endogenous telomerase activity, which is highly regulated during both T-cell development and activation.[15] Telomerase RNA expression and enzymatic activities are transiently up-regulated by T cells upon activation through T-cell–receptor (TCR) ligation in the presence of appropriate costimulatory signals.[16] Despite the fact that forced expression of hTERT results in immortality, the endogenous telomerase activity is insufficient to prevent telomere erosion in vitro and in vivo[5,6,17-20] and does not confer immortality to T cells in vitro. This raises the question whether endogenous telomerase plays a role in the lifespan of human T cells. To investigate this, we used a dominant-negative (DN)–hTERT mutant, which was shown to inhibit endogenous telomerase activity in tumor cells, most likely by competing with hTERT for limiting amounts of telomerase RNA. In tumor cells, expression of DN-hTERT led to an enhanced degree of telomere erosion upon proliferation.[21,22] We examined the consequences of inhibition of endogenous hTERT activity in human T cells by forced expression of 2 different DN versions of the hTERT gene (DN-hTERT). We show here that T cells transduced with DN-hTERT have a shorter

From the Terry Fox Laboratory, British Columbia Cancer Agency and University of British Columbia, Vancouver, Canada; Institut National de la Santé et de la Recherche Médicale (INSERM), Montpellier, France; and the Division of Immunology, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands.

Submitted July 8, 2002; accepted April 2, 2003. Prepublished online as *Blood* First Edition Paper, April 10, 2003; DOI 10.1182/blood-2002-07-2015.

Supported by grant AI29524 from the National Institutes of Health and by a grant from the National Cancer Institute of Canada with funds from the Terry Fox Run. A.R. is funded by a grant from the Deutsche Forschungsgemeinschaft. R.M.L. is supported by a grant from the Dutch Cancer Society (NKI 99-2048).

Reprints: Hergen Spits, Department of Cell Biology & Histology, Amsterdam Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands; e-mail: hergen.spits@amc.uva.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

lifespan than untransduced cells and accumulate cytogenetic abnormalities, which indicates that endogenous telomerase activity is involved in regulating the lifespan of primary human T cells. Strikingly, DN-hTERT expression led to loss of detectable telomere repeats on multiple chromosomes as well as chromosome fusions. However, no generalized telomere shortening was observed, suggesting a role of endogenous telomerase activity in the repair of critically short telomeres. In T cells after extensive in vitro expansion and in freshly isolated memory and naive T cells, we observed that hTERT transcription upon activation decreased with the replicative age of the cells. This decrease correlated with decreased levels in c-myc expression. These results demonstrate the importance of endogenous telomerase activity for the lifespan of telomerase-positive somatic cells and provide a plausible explanation for the limited lifespan of T cells.

Materials and methods

Retroviral constructs

The hTERT retroviral construct hTERT–enhanced green fluorescence protein (GFP) was generated by insertion of hTERT cDNA into the polylinker of LZRS-linker internal ribosomal entry site (IRES)–GFP, as described.[13] Amphotropic retrovirus was made by transfection of the retroviral construct into ΦNX-A cells. DN-hTERT (Asp712Ala, Val713Ile), kindly provided by Dr Robert Weinberg (Massachusetts Institute of Technology [MIT], Boston), was generated by substitution of the aspartic acid and valine residues at positions 712 and 713 in the third reverse transcriptase (RT) motif of hTERT with alanine and isoleucine, respectively, as described.[21] The DN-hTERT (Asp712Ala, Val713Ile) cDNA was inserted into the polylinker of retroviral vector LZRS-IRES-GFP to enable retrovirus production.

DN-hTERT (Asp868Ala, Asp869Ala) complementary DNA, kindly provided by Dr Lea Harrington (University of Toronto, ON, Canada) was generated by substitution of the aspartic acid residues at position 868 and 869 with alanine residues.[22] DN-hTERT (Asp868Ala, Asp869Ala) or the full-length hTERT (cDNA kindly provided by Dr Robert Weinberg) were inserted into a murine stem cell virus (MSCV)–based retroviral vector containing the gene for GFP (Clontech, Palo Alto, CA) under the control of phosphoglycerate kinase 1 (PGK) promoter.[23] Helper-free retrovirus, pseudotyped with the gibbon ape leukemia virus (GALV) envelope for efficient infection of human cells, was generated using PG13 packaging cells.[24]

Isolation of human CD4+ and CD8+ T cells

The Netherlands Cancer Institute's institutional review board approved these studies. Informed consent was provided according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy individuals by density centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). PBMCs were stained with CD4-PE (phycoerythrin; Becton Dickinson, San Jose, CA) and CD8-FITC (fluorescein isothiocyanate; Becton Dickinson). $CD4^+$ or $CD8^+$ cells were sorted by fluorescence-activated cell sorting (FACStar Plus, Vantage SE: Becton Dickinson) and cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% human serum (HS) supplemented with phytohemagglutinin (PHA; Gibco), 100 U/mL recombinant human interleukin 2 (rhIL-2; Roche, Nutley, NJ) and 0.5×10^6/mL irradiated (30 Gy) allogeneic mononuclear feeder cells. The cultures were stimulated weekly with the feeder cells and PHA until sufficient cells were obtained for transduction. The number of population doublings (PDs) was calculated from the average cell count using the following equation: $PD = {}^{10}\log(\text{number of cells counted after expansion}) - {}^{10}\log(\text{number of cells seeded})/{}^{10}\log 2$.

Human T-cell clones

The human $CD4^+$ T-cell clones MoT-72 and MoT-81, specific for the tetanus toxoid peptides (941-960) and (16-35), respectively, were generated from the PBMCs of a healthy individual vaccinated with tetanus toxoid. The human $CD4^+$ T-cell clone BOY JF161 was obtained from $CD4^+$ T cells isolated from a skin biopsy taken from a healthy donor following sensitization and challenge with dinitrochlorobenzene.[25]

Retrovirus-mediated hTERT transduction in T cells and monitoring of cell growth

$CD4^+$ or $CD8^+$ T cells were stimulated with PHA (Gibco) and irradiated allogeneic mononuclear feeder cells prior to transduction. T cells were transduced with retroviral supernatant using 24-well plates coated with fibronectin fragments as described.[26,27] $CD4^+$ T-cell clone MoT-72 was transduced on day 2 after stimulation followed by a second transduction overnight with retrovirus encoding DN-hTERT (Asp712Ala, Val713Ile)–GFP, full-length hTERT-GFP, or control GFP only. Transduction efficiency ranged from 15% to 35%. The bulk of transduced and untransduced cells was cultured by weekly stimulation with a feeder cell mixture consisting of irradiated allogeneic PBMCs (40 Gy), the Epstein-Barr virus (EBV) B-cell line JY (80 Gy), 100 ng/mL PHA and 40 U/mL rhIL-2 for 4 months.[28] All cultures were maintained under equal conditions and the number of population doublings was calculated weekly. The percentage GFP-positive cells in the bulk cultures of DN-hTERT (Asp712Ala, Val713Ile)–GFP, full-length hTERT-GFP, or control GFP-transduced cells and untransduced cells was measured weekly by FACS analysis. Polyclonal T cells, as well as $CD8^+$ T-cell clones obtained by fluorescence-activated single cell sorting (FACStar Plus; Becton Dickinson) were transduced with the DN-hTERT (Asp868Ala, Asp869Ala), full-length hTERT, or control GFP-encoding retrovirus. These retroviruses were all produced as GALV envelope pseudotyped retrovirus. The transduction procedure was repeated on 2 consecutive days. The efficiency of transduction ranged from 1% to 40%. After transduction, cells were expanded for 5 to 6 days and then sorted for GFP expression. DN-hTERT (Asp868Ala, Asp869Ala)–GFP, full-length hTERT-GFP, or control GFP-positive cells were mixed with untransduced T cells in a 50%:50% ratio and cultured for 55 to 65 days. The percentage of GFP-positive cells was measured during the culture to estimate the expansion of the DN-hTERT (Asp868Ala, Asp869Ala)–expressing cells compared with untransduced T cells.

Limiting dilution of GFP-positive cells of clone MoT-72 transduced with DN-hTERT (Asp712Ala, Val713Ile)–GFP, full-length hTERT-GFP, or control GFP was performed by FACStar Plus (Becton Dickinson) in 100-, 30-, 10-, 3-, or 1-cell-per-well densities in round-bottom 96-well plates. Sorted cells were cultured by biweekly stimulation with the feeder cell mixture and rhIL-2 until growing cultures were visible for cloning efficiency determination. The cloning efficiency was calculated as follows: in a negative linear regression curve, the number of cells seeded per well (x-axis) was plotted as a function of the fraction of negative wells (y-axis). The x-value corresponding to a y-value of 0.37 was determined from the graph and indicates the number of cells of which one cell grew out as a clone. This value was recalculated as a percentage indicating the cloning efficiency. Subclones were analyzed for GFP expression and cultured by weekly stimulation with the feeder cell mixture and rhIL-2.

TRAP assay

Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) assay using an end-labeled telomerase substrate (TS) primer as described.[29] DN-hTERT (Asp868Ala, Asp869Ala)–GFP, hTERT-GFP, or control GFP-transduced T cells were sorted 5 days after transduction and cell extracts were obtained from 1×10^5 T cells stimulated with 1 μg/mL PHA, 100 U/mL rhIL-2, and irradiated feeder cells for 2, 4, or 6 days. Cells of the erythroid-myeloid cell line K562 were tested as a positive control cell line for telomerase expression. Control cultures containing feeder cells only were tested to exclude the presence of telomerase activity in the irradiated feeder cells. Elongation of the TS primer by telomerase was performed at room temperature for 30 minutes, and the products were

amplified by 30 cycles of polymerase chain reactions (PCR) at 95°C for 60 seconds, 50°C for 45 seconds, and 72°C for 60 seconds using the anchored return primer. The amplified products were resolved on a 12% polyacrylamide gel and visualized by a phosphoimaging system (Storm 820; Molecular Dynamics, Sunnyvale, CA).

RT-PCR

$CD4^+$ T-cell clones were stimulated with anti-CD3 mAb SPV-T3b[30] and anti-CD28 mAb B-T3 (a kind gift from Dr John Wijdenes, Diaclone, Besançon, France) in the presence of 20 U/mL rhIL-2. Cell pellets of 10^5 cells were snap frozen at day 0, 1, 3, 5, 7, and 11. RNA was isolated from the cell pellets using the RNAeasy kit (Qiagen, Crawley, West Sussex, United Kingdom) and eluted in a volume of 50 μL. RNA (12 μL) was transcribed into cDNA using Superscript II reverse transcriptase (GIBCO-BRL, Life Sciences; Breda, The Netherlands) according to the manufacturer's protocol. PCR reactions were performing using primers 5′-GACACAAACATGATTCAAATCCCTGA-3′ and 5′-TATGGACAGGACTGAACGTCTTGC-3′ to detect hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression for cDNA quantification. Endogenous hTERT expression was detected by primer hTERT 3, 5′-CCTCAGACTCCCAGCGGTGC-3′, which anneals in the nontranslated region of hTERT mRNA that is absent in the ectopic hTERT construct in combination with primer hTERT 5, 5′-CTGCAGGCGTACAGGTTTCACG-3′. PCR reactions of primer hTERT 5 and primer IRES 3, 5′-GAGAGGGGCGGAATTTACGTAG-3′, which anneals to the IRES sequence present in the hTERT retroviral construct, were used to detect ectopic hTERT expression. PCR reactions were run at 94°C for 4 minutes followed by 30 cycles of 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 1 minute. For endogenous hTERT detection 35 cycles were run to detect low levels of hTERT expression. PCR reactions specific for c-myc were performed using primers, 5′-TCGGATTCTCTGCTCTCCTC-3′ and 5′-TTCCGCAACAAGTCCTCTTC-3′, and run in parallel with HPRT PCR reactions at 94°C for 4 minutes followed by 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. PCR products were visualized on a 1.5% agarose gel by ethidium bromide staining. PCR product bands were quantified relative to HPRT expression using the Phoretix 1D Advanced imager software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). PCR reactions were performed on serial 2-fold dilutions of the cDNA samples. Relative levels of gene expression were calculated from the linear part of the dilution curve, normalized to the HPRT expression level.

Real-time PCR

PBMCs from 3 donors were incubated with PE-conjugated anti-CD27 mAbs (Becton Dickinson) and Cy-5–conjugated anti-CD45RA mAbs (Becton Dickinson), washed, and $CD27^+CD45RA^+$ (naive) and $CD27^+CD45RA^-$ (memory) T cells were sorted by fluorescence-activated cell sorting. Sorted cells, which were of more than 95% purity and consisted of equal numbers of $CD28^+$ T cells, were stimulated with anti-CD3 and anti-CD28 mAb–coated beads (Dynabeads CD3/CD28 T-cell expander; Dynal, Oslo, Norway) and 20 U/mL rhIL-2 or cultured in rhIL-2 alone for 2 days, and snap frozen at −80°C as pellets of 2×10^5 cells. RNA was extracted from the cells using the RNAeasy kit and eluted in a volume of 50 μL. RNA (11 μL) was transcribed into cDNA by 5 U/μL reverse transcriptase (Superscript II; Invitrogen, Breda, The Netherlands) using 12.5 μM random hexamers (Applied Biosystems, Foster City, CA), in the presence of 1 mM deoxynucleoside triphosphates (dNTPs), 0.01 M dithiothreitol (DTT), and 1 U/μL RNAse inhibitor (Applied Biosystems), in a volume of 40 μL. Per reaction, 5 μL cDNA was amplified in a Sequence Detector (Perkin Elmer, Norwalk, CT) using 5′ primer 5′-TTTTCTACCGGAAGAGTGTCTGG-3′, 3′ primer 5′-GCTTCCCGATGCTGCCT-3′, and carboxyfluorescein (FAM; Applied Biosystems)–labeled probe 5′-TTGCAAAGCATTGGAATCAGACAGC ACT-3′ for hTERT detection. hTERT PCR reactions were run in triplicate. Expression of the housekeeping genes, human TATA box–binding protein (TBP), glyceraldehyde phosphate dehydrogenase (GAPDH), and β-actin (Applied Biosystems) were tested in duplicate on the cDNA samples. Expression of the housekeeping gene porphobilinogen deaminase (PBGD) was tested using 5′ primer 5′-ACGATCCCGAGACTCTGC-3′, 3′ primer 5′-GCACGGCTACTGGCACACT-3′, and VIC-labeled probe 5′-CCTGAGGCACCTGGAAGGAGGCTG-3′ (kindly provided by C. Bosch and Dr E. Robanus-Maandag, The Netherlands Cancer Institute, Amsterdam). A 10-fold dilution range of cDNA obtained from the telomerase-expressing B-cell line JY was tested in duplicate to define a standard curve for the expression level. Standard curves were linear with a correlation coefficient higher than 0.998 and a negative slope of more than 3.5 to ensure reliable quantitative amplification detection. Gene expression of the samples was analyzed in the linear range of the amplification plot using the Sequence Detector System software (Perkin Elmer) and expressed as a value relative to the standard curve.

Telomere analysis by quantitative FISH on metaphase chromosomes

Cloned $CD4^+$ T lymphocytes that had undergone approximately 24 PDs were transduced with DN-hTERT (Asp868Ala, Asp869Ala)–GFP, full-length hTERT-GFP, or control GFP. Cells of an approximate age of 45 PDs were stimulated with 1 μg/ml PHA, 100 U/ml rhIL-2, and 0.5×10^6/mL irradiated allogeneic feeder cells for 4 to 5 days before addition of colcemid for 1 hour. Cells were then treated with hypotonic KCl for 50 minutes at 37°C and fixed in methanol–acetic acid. Quantitative fluorescence in situ hybridization (Q-FISH) on metaphase chromosomes was performed with Cy-3–labeled $(CCCTAA)_3$ PNA probe and subsequent quantitative analysis of digital images as previously described.[31,32] Briefly, slides were observed with an Axioplan microscope (Zeiss, Thornwood, NY) equipped with a charged coupled device (CCD) camera. Separate images were captured for DAPI (4,6 diamidino-2-phenylindole) and Cy-3 and subjected to telomere fluorescence measurements using TFL-Telo software.[33] Metaphases were analyzed for the presence of chromosomal abnormalities, such as dicentric chromosomes, and for the number of detectable telomeres per chromosome. Individual telomere length was quantified by the level fluorescence intensity of each telomere spot, expressed in telomere fluorescence units (TFUs).

Results

Dominant-negative hTERT expression decreases the lifespan of human $CD4^+$ T cells

We tested the contribution of endogenous hTERT to the lifespan of human T cells by expression of 2 different DN mutants of the hTERT gene, DN-hTERT (Asp868Ala, Asp869Ala) and DN-hTERT (Asp712Ala, Val713Ile), which both inhibit telomerase activity in tumor cells.[21,22] In human polyclonal T cells isolated from PBMCs and transduced with DN-hTERT (Asp868Ala,

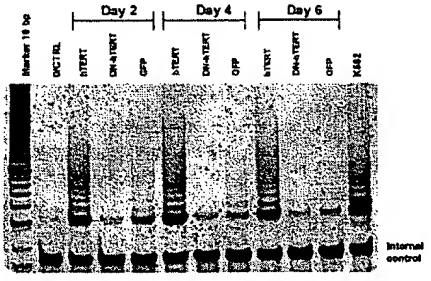

Figure 1. Inhibition of telomerase activity by ectopic DN-hTERT expression. Telomerase activity in a culture of polyclonal T cells isolated from donor PBMCs transduced with DN-hTERT (Asp868Ala, Asp869Ala)–GFP, hTERT-GFP, or control GFP after 2, 4, and 6 days of stimulation. Telomerase activity was detected in control GFP-transduced cells 2 to 4 days after stimulation and was absent in the DN-hTERT–transduced cells. In hTERT-transduced cells telomerase activity was present at all time points analyzed. Each lane shows the telomerase activity present in 2000 cells analyzed by the TRAP assay. K562 cells were tested as a positive control for telomerase activity. Results are representative of 3 experiments.

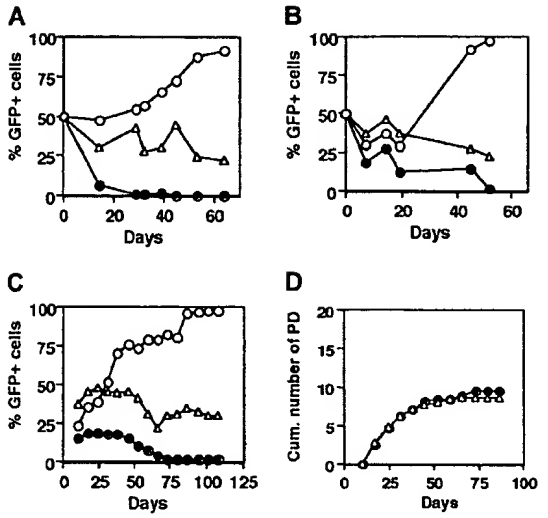

Figure 2. Dominant-negative hTERT-transduced T cells disappear early from bulk cultures. (A) Human polyclonal CD4+ T cells transduced with either DN-hTERT (Asp868Ala, Asp869Ala)–GFP (●), hTERT-GFP (○), or control GFP (△) were mixed with untransduced cells in a 50%:50% ratio and cultured in parallel for 53 days to determine the lifespan compared with the untransduced T cells. The graph shows the percentage of GFP-positive cells during the culture. Results are representative of 3 experiments. (B) CD8+ T-cell clone transduced and cultured as described in panel A. Results are representative of 2 experiments. (C) CD4+ T-cell clone MoT-72 was transduced with either DN-hTERT (Asp712Ala, Val713Ile)–GFP (●), hTERT-GFP (○), or control GFP (△), and the percentage of GFP-positive cells was measured weekly during the culture. The graph shows the percentage GFP-positive cells during the culture. Transduction efficiencies at the onset of the cultures were as follows: hTERT-GFP, 20%; GFP control, 35%; and DN-hTERT–GFP, 15%. Results are representative of experiments with 6 different CD4+ T-cell clones. Panels A-C show that in both CD4+ and CD8+ T-cell cultures, ectopic expression of DN-hTERT decreased the lifespan compared with untransduced T cells. (D) Cumulative number of population doublings of the untransduced GFP-negative cells in the mixed culture containing DN-hTERT (●) or GFP-transduced T cells (△). The graph shows that the untransduced T cells were growing equally well in both mixed cultures, indicating that the decrease in percentage DN-hTERT–GFP–expressing T cells in DN-hTERT-GFP–transduced cultures was not due to an enhanced proliferation of the untransduced T cells compared with untransduced T cells in the control GFP-transduced cultures.

Asp869Ala)–GFP, hTERT-GFP, or the empty vector–encoding GFP only, the telomerase activity upon stimulation was reduced in DN-hTERT–transduced cells compared with control GFP-transduced cells (Figure 1). Polyclonal CD4+ T cells that had been cultured for a short period of time before transduction with DN-hTERT (Asp868Ala, Asp869Ala)–GFP were cultured in a 50%:50% ratio with untransduced cells and the percentage of GFP-expressing cells was measured during the culture (Figure 2A). The percentage of DN-hTERT–GFP–positive cells decreased over time during the culture and disappeared from the culture within 30 to 50 days, demonstrating that these cells did not expand and died earlier than the untransduced cells in the same culture. Similar results were obtained in experiments of cloned CD8+ cells transduced with DN-hTERT–GFP, indicating that DN-hTERT expression also led to early death of cells of an at least 20-PD higher replicative age acquired during T-cell cloning (Figure 2B). In control cultures of cells transduced with the empty GFP-containing vector, the percentage of GFP-positive cells did not change over time. In contrast, hTERT-GFP–transduced cultures showed an accumulation of cells expressing the hTERT-GFP gene. The limiting effect of DN-hTERT expression on the lifespan of human T cells was confirmed with a second DN mutant of the hTERT gene, DN-hTERT (Asp712Ala, Val713Ile), expressed in the CD4+ T-cell clone MoT-72. MoT-72 cells transduced with DN-hTERT (Asp712Ala, Val713Ile)–GFP, were cleared from mixed cultures of transduced and untransduced cells within 75 days (Figure 2C). These results indicate that, compared with untransduced or control GFP-transduced T cells, expression of a dominant-negative mutant of the hTERT gene confers a growth disadvantage in both CD4+ and CD8+ T cells.

The observed growth disadvantage following DN-hTERT expression was further investigated by limiting dilution experiments of sorted DN-hTERT (Asp712Ala, Val713Ile)–GFP expressing cells. In 2 independent experiments, the efficiency of subcloning DN-hTERT (Asp712Ala, Val713Ile)–GFP–transduced cells of clone MoT-72 was lower than the subcloning efficiency of untransduced cells (Table 1), or cells transduced with hTERT-GFP, indicating that many DN-hTERT–transduced T cells could not expand another 20 PDs in culture to establish a subclone. The few subclones that were obtained from the cloning of DN-hTERT (Asp712Ala, Val713Ile)–GFP–expressing cells all died soon after cloning at lower population doublings than control GFP-transduced subclones. No GFP-negative clones were obtained in any experiments. Since the DN-hTERT–expressing subclones expressed low levels of GFP, these subclones had probably arisen by selective outgrowth

Table 1. Subcloning efficiency of DN-hTERT (Asp712Ala, Val713Ile)–GFP, hTERT-GFP, or control GFP-transduced MOT-72 CD4+ T cells

	MOT-GFP		DN-hTERT-GFP		hTERT-GFP	
Cells/well	Clones*	% total	Clones*	% total	Clones*	% total
Experiment 1						
100	48/48	100	35/40	87	48/48	100
30	40/48	83	13/40	33	41/48	85
10	14/48	29	1/48	2	8/48	17
3	9/48	19	1/48	2	1/48	2
1	5/96	5	2/96	2	8/96	8
Cloning efficiency, %	4.4	NA	1.7†	NA	4.2	NA
Experiment 2						
100	34/48	70	27/48	56	47/48	98
30	11/48	23	6/48	13	25/48	52
10	0/48	0	2/48	4	6/48	13
3	1/48	2	0/48	0	3/48	6
1	0/96	0	0/96	0	0/96	0
Cloning efficiency, %	1.2	NA	0.8†	NA	2.7	NA

NA indicates not applicable.

*Number of wells with growing cells/total number of wells seeded.

†All clones died shortly after cloning.

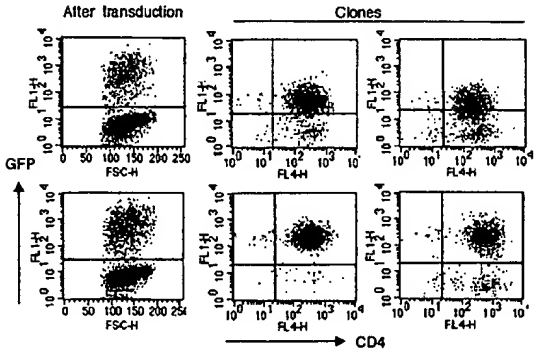

Figure 3. Subclones of DN-hTERT–transduced T cells display lower DN-hTERT gene expression. The level of GFP expression is decreased in DN-hTERT–GFP–transduced clones. Two clones isolated from limiting dilution of MoT-72 transduced with DN-hTERT (Asp712Ala, Val713Ile)–GFP (top middle and top right), displayed a lower GFP expression than the average GFP level expressed by the bulk culture right after transduction (top left). Graphs are representative of the GFP expression by all DN-hTERT clones obtained. All DN-hTERT (Asp712Ala, Val713Ile)–GFP–expressing clones died after cloning. The GFP expression remained high in clones isolated from cloning of MoT-72 transduced with hTERT-GFP (bottom middle and bottom right). This level of GFP expression was equal to the GFP expression level after transduction (bottom left). Graphs are representative of 16 hTERT-GFP–expressing clones analyzed.

of cells with a relatively low transgene expression, in which inhibition of endogenous telomerase activity is less efficient (Figure 3). Thus, cells with higher levels of GFP and DN-hTERT expression had selectively disappeared from the cultures at earlier time points. In contrast, all subclones obtained from hTERT-GFP or control GFP-transduced cells expressed GFP at levels comparable to those right after transduction, indicating that, in these cultures, cells with a high level of GFP expression had no selective disadvantage during cloning (Figure 3).

Cytogenetic abnormalities in DN-hTERT–transduced cells

Next, we studied the effect of endogenous telomerase inhibition on chromosomal stability and telomere length by Q-FISH (Tables 2 and 3; Figure 4). For this purpose, we transduced the progeny of a single $CD4^+$ T cell with retroviral vectors containing GFP, GFP-hTERT, or GFP-DN (Asp868Ala, Asp869Ala)–hTERT. Figure 4 shows preparations of metaphase chromosomes of sorted GFP-positive cells hybridized with Cy-3–labeled $(CCCTAA)_3$ peptide nucleic acid probe specific for telomeric DNA. Metaphase chromosomes of cells that were transduced with the GFP-DN (Asp868Ala, Asp869Ala)–hTERT display a higher frequency of chromosomes without detectable telomeric DNA than those in control GFP-transduced cells. On average, only 3.5 telomeres were detectable per chromosome in DN (Asp868Ala, Asp869Ala)–hTERT–transduced cells, whereas all 4 telomeres per chromosome were detected on most chromosomes in control GFP-transduced cells or hTERT-transduced cells (Tables 2-3). Expression of DN-hTERT significantly decreased the number of detectable telomeres per chromosome compared with control GFP-transduced cells ($P < .001$) or with hTERT-GFP–transduced cells ($P < .001$; Table 3).

In addition, several dicentric chromosomes were observed in the GFP-DN (Asp868Ala, Asp869Ala)–hTERT–transduced cells, which were absent in the GFP- or hTERT-GFP–transduced cells. No telomeric DNA was detected at the junction site in the dicentric chromosomes. The chromosome fusions observed in GFP-DN (Asp868Ala, Asp869Ala)–hTERT–transduced cells involved fusions of chromosomes 1, 7, and 6 with chromosome 20, 22, and 10, respectively (Figure 4; Table 2). Q-FISH analysis revealed that the average length of the telomeres in DN (Asp868Ala, Asp869Ala)–hTERT-GFP–transduced cells was comparable to control GFP- or hTERT-GFP–transduced cells. Strikingly, Q-FISH analysis also revealed that DN-hTERT expression increased the frequency of very short telomeres that are undetectable by in situ hybridization (Figure 5). Most likely some of the chromosomes with undetectable telomere repeats in DN-hTERT expressing cells underwent chromosome end-to-end fusions. The observation that inhibition of endogenous telomerase activity did not lead to a decrease in the average telomere length, but instead to an increase in the number of undetectable telomeres and to the occurrence of chromosome fusions, suggests a role of endogenous telomerase activity in the repair of critically short telomeres rather than in mediating telomere elongation in all chromosomes.

Activation-induced endogenous hTERT expression in human T cells decreases with age

The results described here indicate that endogenous hTERT expression is important for the survival of human T cells, raising the question as to why this is not sufficient to confer immortality to the T cells. One possibility is that the limited lifespan of T cells might result from a decrease in the expression of the telomerase complex. We therefore determined the endogenous hTERT expression levels in T cells of different replicative age by comparing the up-regulation of endogenous hTERT expression in early cultures with that in long-term–cultured T cells.

To determine variations in the expression of endogenous hTERT in long-term cultures of hTERT-transduced cells, we developed an RT-PCR analysis using sets of primers that discriminate between endogenous hTERT and transduced hTERT expression. In Figure 6A the expression of endogenous hTERT, 1 to 7 days after antibody-mediated activation in an early culture of the $CD4^+$ T-cell clone MoT-81 (20 PD), is shown. Both wild-type and hTERT-transduced MoT-81 cells with identical replicative age show hTERT expression one day after stimulation, which is no longer detectable after 5 days. Furthermore, these results show that ectopic hTERT expression did not affect the up-regulation of endogenous hTERT expression following TCR/CD3-mediated activation.

In contrast, in a long-term culture of an hTERT-transduced clone that had undergone more than 60 PDs after its generation, no endogenous hTERT could be detected (Figure 6B). Wild-type cells at an earlier time point (36 PDs) of culture were still able to induce endogenous hTERT expression one day after stimulation with anti-CD3 and anti-CD28 monoclonal antibodies (Figure 6B). Both wild-type and hTERT-transduced cells proliferated extensively following stimulation (data not shown), excluding the possibility that the absence of endogenous hTERT expression in the latter cells was due to the lack of T-cell activation. Taken together, these

Table 2. Chromosomal abnormalities in dominant-negative hTERT-transduced $CD4^+$ T cells

DNA construct used for $CD4^+$ T-cell transduction	No. of metaphases analyzed	No. of metaphases with dicentric chromosomes
GFP	28	0
DN-hTERT-GFP	16	3*
hTERT-GFP	20	0

*Chromosome end-to-end fusions found: (1q;20q) (7p;22p) (6q;10p).

Table 3. Loss of detectable telomeres in dominant-negative hTERT-transduced CD4+ T cells

DNA construct used for CD4+ T-cell transduction	Metaphases analyzed	Chromosomes analyzed	Telomere spots*	Mean no. of spots/chromosome	SD	Significance† compared with GFP
GFP	16	735	2818	3.83	0.11	NA
DN-hTERT-GFP	16	733	2498	3.42	0.17	<0.001
hTERT-GFP	16	731	2861	3.91	0.09	0.038

NA indicates not applicable.
*Total number of telomere spots measured by Q-FISH.
†Significance of the difference in numbers of detectable telomeres, compared with control GFP-transduced T cells, was tested in a Student *t* test.

results show that upon long-term culture, T cells lose the ability to increase endogenous hTERT expression upon activation.

Next, we investigated what caused the decreased hTERT expression in long-term–cultured T cells. The c-myc transcription factor directly activates hTERT transcription by dimerizing with the Max protein and binding to E-box binding sites that are present in the hTERT promoter.[34] We analyzed c-myc expression upon activation by RT-PCR analysis with primers that are specific for c-myc (Figure 7). In resting T cells from both long-term and short-term cultures, low levels of c-myc expression were detected (Figure 7 day 0). One day after stimulation with anti-CD3 and anti-CD28 mAbs, the c-myc expression in the relatively young T cells increased more than 70-fold compared with levels of c-myc expression in unstimulated cells (Figure 7A day 0), with a somewhat lower increase (27-fold) after 3 days (Figure 7A). In the long-term–cultured T cells of the same clone, one day after stimulation the activated cells expressed only 7 times more c-myc RNA than unstimulated cells (Figure 7B). Taken together, these results show that during long-term culture T cells have a strongly reduced capacity to up-regulate hTERT expression upon activation, which is correlated with a strongly reduced up-regulation of c-myc.

The loss of hTERT expression upon activation may also occur upon expansion in vivo. Memory and effector T cells have expanded upon antigen encounter in vivo and on average have divided considerably more frequently than naive T cells of the same donor. We compared the level of hTERT expression upon stimulation in naive and memory cells isolated from donor PBMCs by quantitative real-time PCR. T cells were sorted by their expression of CD27 and CD45RA to isolate the $CD27^+CD45RA^+$ naive and $CD27^+CD45RA^-$ memory T-cell population.[35] The percentage of CD28-expressing cells in the sorted naive and memory T-cell population was identical in each donor, and comprised 80% to 90% (data not shown). Following stimulation with anti-CD3 and anti-CD28 antibodies, we observed an up-regulation of hTERT expression in both activated naive and memory T cells compared with the nonactivated cells. However, hTERT expression levels were lower in activated memory cells than in activated naive cells of the same donor (Table 4). These results suggest that expansion of T cells upon antigen encounter in vivo may eventually result in a lower hTERT expression upon activation.

Discussion

We report here that endogenous telomerase activity plays a role in regulating the lifespan of human T cells. Blocking endogenous telomerase activity by ectopic expression of either the DN-hTERT (Asp712Aala, Val713Ile) or the DN-hTERT (Asp868Ala, Asp869Ala) mutant of the hTERT gene was shown to shorten the lifespan of both short-term– and long-term–cultured $CD4^+$ T cells and $CD8^+$ T cells. The observation that endogenous telomerase does not confer immortality to human T cells might imply that endogenous telomerase is not involved in lifespan regulation. However, our results indicate that this assumption is incorrect, since inactivation of endogenous telomerase resulted in a shortened lifespan. Our observation that upon in vitro culture T cells progressively lose their capacity to up-regulate hTERT expression upon in vitro culture suggests that, upon aging, levels of endogenous telomerase are increasingly insufficient to maintain telomere length of more than a few short telomeres. Importantly, memory T cells were found to express less hTERT upon activation than naive cells from the same donor, indicating that a loss of hTERT up-regulating ability may also occur in vivo.

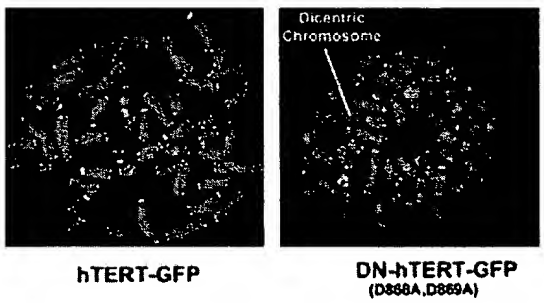

Figure 4. DN-hTERT–transduced cells show chromosomal abnormalities. Q-FISH analysis of metaphase chromosomes isolated from human $CD4^+$ T cells that have undergone approximately 45 PD and were transduced with DN-hTERT (Asp868Ala, Asp869Ala)–GFP or hTERT-GFP at approximately 24 PD. Telomeres are visualized in yellow by hybridization with the Cy-3–labeled $(CCCTAA)_3$ PNA probe. The arrow indicates a dicentric chromosome found in DN-hTERT (Asp868Ala, Asp869Ala)–GFP–transduced cells. Results are representative of 16 metaphases of DN-hTERT–transduced cells and 20 metaphases of hTERT-transduced cells analyzed.

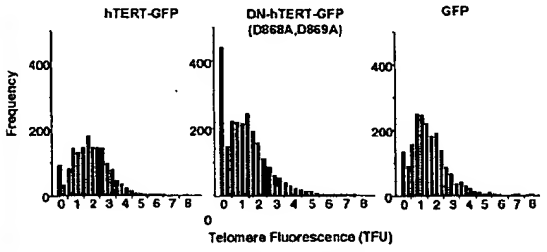

Figure 5. Telomere length analysis of human $CD4^+$ T cells transduced with DN-hTERT. Graphs show the quantification of individual telomere length expressed as the distribution of TFUs of Q-FISH telomere analysis of metaphase chromosomes from human $CD4^+$ T cells that have undergone approximately 45 PD and were transduced with DN-hTERT (Asp868Ala, Asp869Ala)–GFP, hTERT-GFP, or control GFP. Analyses were performed on 16 metaphases of DN-hTERT (Asp868Ala, Asp869Ala)–GFP–transduced $CD4^+$ T cells, 20 metaphases of hTERT-GFP–transduced cells, and 28 metaphases of GFP-transduced cells. The average telomere fluorescence of individual chromosome ends was calculated to be 1.35 TFU for DN-hTERT (Asp868Ala, Asp869Ala)–GFP–transduced cells, 1.92 TFU for hTERT-GFP–transduced cells, and 1.60 TFU for control GFP-transduced cells.

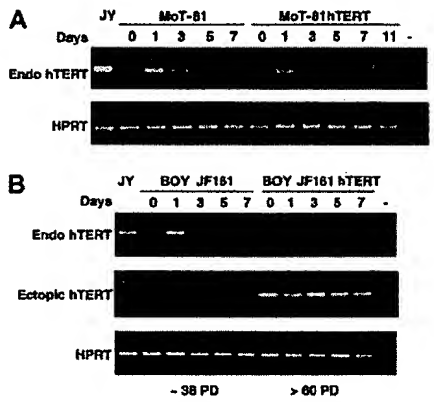

Figure 6. Expression of endogenous hTERT upon stimulation during culture. (A) Wild-type MoT-81 cells and hTERT-transduced cells of identical age were stimulated with anti-CD3 and anti-CD28 mAbs, and the expression of endogenous hTERT at day 0, 1, 3, 5, 7, and 11 was determined by RT-PCR. All samples were tested for HPRT expression to check for equal amounts of RNA. (B) RT-PCR analysis of endogenous and ectopic hTERT expression in wild-type BOY JF161 cells after approximately 36 PD and in hTERT-GFP–transduced cells after more than 60 PD.

T cells that had been transduced with DN-hTERT showed a loss of detectable telomeres at many chromosome ends and a high frequency of dicentric chromosomes. The observed chromosome fusions and chromosome ends without detectable telomeric DNA are similar to the type of abnormalities seen in various cells from late generations of the telomerase mTERC knock-out mice.[36] The dramatic effect of telomerase inhibition on some, but not all, telomeres is compatible with a role of endogenous telomerase in the repair of sporadic telomere attrition. One possibility is that telomerase is essential for the repair of telomeric DNA following damage by oxygen radicals.[37-38] It has been shown that G-rich telomeric DNA is particularly vulnerable to oxidative stress,[39,40] and it has been proposed that such damage is the primary cause of telomere attrition in human cells.[41] Our results suggest that while endogenous telomerase may be essential to repair sporadic telomere shortening, overall telomere shortening and replicative senescence is not prevented.

One explanation is that endogenous telomerase levels are simply insufficient to deal with more than a few critically short telomeres, resulting in a net telomere loss that increases gradually over time. The observation that ectopic expression of hTERT immortalizes human T lymphocytes[13,14] indicates that increased levels of hTERT expression contribute to longevity in T cells. Moreover, the telomerase activity upon hTERT expression may depend on phosphorylation and nuclear translocation of the hTERT protein.[42] Another possibility is that broken ends (perhaps following some processing event) are better substrates for telomerase than natural chromosome ends that may be folded into a T loop,[43] which is underscored by the recently described possible differences in the processing of telomeres.[44] Furthermore, the ability of T cells to up-regulate hTERT expression upon activation may decrease over time, resulting in accelerated telomere erosion in aged T cells.[45]

Our results strengthen the observation of Weng et al[45] of a progressive decrease in telomerase activity at each sequential stimulation in a polyclonal culture of human $CD4^+$ T cells. The loss of telomerase expression upon activation may also occur upon extensive expansion of T cells in vivo. We have shown that memory T cells express lower levels of hTERT when activated ex vivo than naive T cells of the same donor. This decrease in hTERT up-regulation correlates with the greater replicative history of memory T cells compared with naive T cells. However, Weng and coworkers[20] reported that activation-induced telomerase activity in polyclonal $CD4^+$ or $CD8^+$ T cells did not decrease with the donor age, whereas the average telomere length was shorter in older individuals. This suggests that donor age may not reveal the differences in replication of the entire $CD4^+$ or $CD8^+$ T-cell population and that variations in telomerase expression may be more evident between T-cell populations within the same donor. The same authors also reported comparable levels of telomerase activity in activated $CD45RA^+$ naive and $CD45RO^+$ memory T cells.[46] It cannot be excluded, however, that $CD45RA^+$ effector cells were present in the naive T-cell fraction, which may have a lower level of telomerase induction. We have observed that the total $CD45RA^+$ cell fraction in donor PBMCs contained up to 50% $CD45RA^+CD27^-$ effector cells. Therefore, contaminating effector cells may have lowered the average telomerase level that was detected in the $CD45RA^+$ T-cell population and thereby may have concealed possible differences in telomerase activity between activated naive and memory T cells. Moreover, the effect of aging on the telomerase expression was also observed in T cells of patients with X-linked lymphoproliferative disorder, characterized by a primary immunodeficiency disease that leads to an inability to regulate the immune response to EBV. In these patients, the EBV-reactive T cells were found to have greatly reduced telomere lengths, as well as a reduced telomerase activity and proliferative capacity upon stimulation in vitro compared with EBV-reactive T cells of chronically infected control individuals (Dr A. Akbar, personal communication).

The impaired hTERT expression may be caused by the reduced c-myc expression upon activation that we have observed in long-term–cultured T cells, since hTERT is a direct target for c-myc.[47-49] Strikingly, c-myc expression levels of activated T cells of humans of old age is decreased compared with those in young donors and coincided with a reduced proliferative capacity.[50] Transcription of hTERT is tightly regulated by c-myc, by other activators of transcription such as Sp1 and estrogen, as well as by

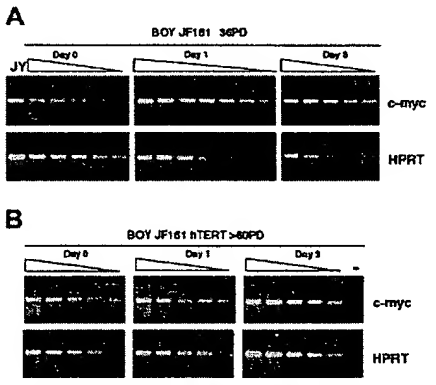

Figure 7. Expression of c-myc upon activation. BOY JF161 T cells (after approximately 36 PD, A) and long-term–cultured BOY JF161-TERT T cells (after more than 60 PD, B) were stimulated with anti-CD3 and anti-CD28 mAbs, and c-myc expression was determined at day 1 and day 3 after stimulation. Day 0 indicates unstimulated cells. c-myc and HPRT PCR reactions were run in parallel on a 1:2 dilution range of the cDNA samples as indicated by the triangles above the lanes. The increase in c-myc expression after activation relative to unstimulated cells (day 0) was quantified by comparing the relative peak intensity of c-myc PCR products at equal intensity of HPRT amplification.

Table 4. Real-time PCR analysis of hTERT expression upon activation in freshly isolated naive and memory T cells

Donor	T-cell population	Nonstimulated	Stimulated	Fold increase in hTERT expression*	hTERT expression relative to naive cells†
1	Naive	0.075‡	8.474	113.3	1
1	Memory	0.042	2.051	49.0	0.43
2	Naive	0.027	3.289	120.0	1
2	Memory	0.011	0.889	78.3	0.65
3	Naive	0.019	1.014	52.4	1
3	Memory	0.015	0.616	41.2	0.79

*Increase in hTERT expression upon activation.
†Up-regulation of hTERT expression relative to naive cells.
‡Values are corrected for the expression of the housekeeping gene human TBP in nonstimulated cells of each population.

repressors of hTERT transcription, such as Mad1, WT1, p53, and MZF-2.[51] Aging of T cells may have an effect on more regulators of hTERT transcription, resulting in a decreased telomerase activity in older cells. The remarkable effect of inhibition and overexpression of telomerase on T-cell growth indicates that telomerase levels in vivo are very tightly controlled. This conclusion is supported by the observation that individuals with a heterozygous defect in telomerase RNA suffer from dyskeratosis congenita, a rare genetic disorder typically resulting in early death form aplastic anemia, cancer, or immune deficiency.[52]

Acknowledgments

We thank Dr Robert Weinberg (MIT, Boston, MA) for the DN-hTERT (Asp712Ala, Val713Ile) cDNA, Dr Lea Harrington (University of Toronto) for the DN-hTERT (Asp868Ala, Asp869Ala) cDNA, Dr Marianne Naspetti for designing hTERT and c-myc PCR primers, and Cathy Bosch and Astrid Bosma for providing real-time PCR primers and probes and their excellent help with the Taqman analyses.

References

1. Effros RB, Pawelec G. Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion? Immunol Today. 1997;18:450-454.
2. Pawelec G, Sansom D, Rehbein A, Adibzadeh M, Beckman I. Decreased proliferative capacity and increased susceptibility to activation-induced cell death in late-passage human CD4+ TCR2+ cultured T cell clones. Exp Gerontol. 1996;31:655-668.
3. Grubeck-Loebenstein B, Lechner H, Trieb K. Long-term in vitro growth of human T cell clones: can postmitotic 'senescent' cell populations be defined? Int Arch Allergy Immunol. 1994;104:232-239.
4. Weng NP, Levine BL, June CH, Hodes RJ. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. Proc Natl Acad Sci U S A. 1995;92:11091-11094.
5. Rufer N, Dragowska W, Thornbury G, Roosnek E, Lansdorp PM. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. Nat Biotechnol. 1998;16:743-747.
6. Vaziri H, Schachter F, Uchida I, et al. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet. 1993;52:661-667.
7. Rufer N, Brummendorf TH, Kolvraa S, et al. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. J Exp Med. 1999;190:157-167.
8. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;345:458-460.
9. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colorectal carcinoma and with ageing. Nature. 1990;346:866-868.
10. McEachern MJ, Krauskopf A, Blackburn EH. Telomeres and their control. Annu Rev Genet. 2000;34:331-358.
11. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. Science. 1998;279:349-352.
12. Vaziri H, Benchimol S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol. 1998;8:279-282.
13. Hooijberg E, Ruizendaal JJ, Snijders PJ, Kueter EW, Walboomers JM, Spits H. Immortalization of human CD8+ T cell clones by ectopic expression of telomerase reverse transcriptase. J Immunol. 2000;165:4239-4245.
14. Rufer N, Migliaccio M, Antonchuk J, Humphries RK, Roosnek E, Lansdorp PM. Transfer of the human telomerase reverse transcriptase (TERT) gene into T lymphocytes results in extension of replicative potential. Blood. 2001;98:597-603.
15. Weng NP, Levine BL, June CH, Hodes RJ. Regulated expression of telomerase activity in human T lymphocyte development and activation. J Exp Med. 1996;183:2471-2479.
16. Liu K, Schoonmaker MM, Levine BL, June CH, Hodes RJ, Weng NP. Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes. Proc Natl Acad Sci U S A. 1999;96:5147-5152.
17. Bodnar AG, Kim NW, Effros RB, Chiu CP. Mechanism of telomerase induction during T cell activation. Exp Cell Res. 1996;228:58-64.
18. Pan C, Xue BH, Ellis TM, Peace DJ, Diaz MO. Changes in telomerase activity and telomere length during human T lymphocyte senescence. Exp Cell Res. 1997;231:346-353.
19. Burns JB, Lobo ST, Bartholomew BD. In vivo reduction of telomere length in human antigen-reactive memory T cells. Eur J Immunol. 2000;30:1894-1901.
20. Son NH, Murray S, Yanovski J, Hodes RJ, Weng N. Lineage-specific telomere shortening and unaltered capacity for telomerase expression in human T and B lymphocytes with age. J Immunol. 2000;165:1191-1196.
21. Hahn WC, Stewart SA, Brooks MW, et al. Inhibition of telomerase limits the growth of human cancer cells. Nat Med. 1999;5:1164-1170.
22. Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev. 1999;13:2388-2399.
23. Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. Gene Ther. 1994;1:136-138.
24. Miller AD, Garcia JV, von Suhr N, Lynch CM, Wilson C, Eiden MV. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. J Virol. 1991;65:2220-2224.
25. Lecart S, Boulay V, Raison-Peyron N, et al. Phenotypic characterization of human CD4+ regulatory T cells obtained from cutaneous dinitrochlorobenzene-induced delayed type hypersensitivity reactions. J Invest Dermatol. 2001;117:318-325.
26. Heemskerk MH, Hooijberg E, Ruizendaal JJ, et al. Enrichment of an antigen-specific T cell response by retrovirally transduced human dendritic cells. Cell Immunol. 1999;195:10-17.
27. Pollok KE, Hanenberg H, Noblitt TW, et al. High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments. J Virol. 1998;72:4882-4892.
28. Yssel H, De Vries JE, Koken M, Van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Methods. 1984;72:219-227.
29. Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res. 1997;25:2595-2597.
30. Spits H, Keizer G, Borst J, Terhorst C, Hekman A, de Vries JE. Characterization of monoclonal antibodies against surface molecules associated with cytotoxic activity of natural and activated killer cells and cloned CTL lines. Hybridoma. 1983;2:423-437.
31. Zijlmans JM, Martens UM, Poon SS, et al. Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. Proc Natl Acad Sci U S A. 1997;94:7423-7428.
32. Lansdorp PM, Verwoerd NP, van de Rijke FM, et al. Heterogeneity in telomere length of human chromosomes. Hum Mol Genet. 1996;5:685-691.
33. Poon SS, Martens UM, Ward RK, Lansdorp PM. Telomere length measurements using digital fluorescence microscopy. Cytometry. 1999;36:267-278.

34. Cerni C. Telomeres, telomerase, and myc: an update. Mutat Res. 2000;462:31-47.
35. Hamann D, Roos MT, van Lier RA. Faces and phases of human CD8 T-cell development. Immunol Today. 1999;20:177-180.
36. Blasco MA, Lee HW, Hande MP, et al. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell. 1997;91:25-34.
37. Lansdorp PM. Repair of telomeric DNA prior to replicative senescence. Mech Ageing Dev. 2000; 118:23-34.
38. Luiten RM, Pène J, Yssel H, Spits, H. Ectopic hTERT expression extends the life span of human CD4+ helper and regulatory T-cell clones and confers resistance to oxidative stress–induced apoptosis. Blood. 2003;101:4512-4519.
39. Henle ES, Han Z, Tang N, Rai P, Luo Y, Linn S. Sequence-specific DNA cleavage by Fe^{2+}-mediated fenton reactions has possible biological implications. J Biol Chem. 1999;274:962-971.
40. Honda S, Hjelmeland LM, Handa JT. Oxidative stress—induced single-strand breaks in chromosomal telomeres of human retinal pigment epithelial cells in vitro. Invest Ophthalmol Vis Sci. 2001; 42:2139-2144.
41. von Zglinicki T, Pilger R, Sitte N. Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radic Biol Med. 2000;28:64-74.
42. Liu K, Hodes RJ, Weng N. Cutting edge: telomerase activation in human T lymphocytes does not require increase in telomerase reverse transcriptase (hTERT) protein but is associated with hTERT phosphorylation and nuclear translocation. J Immunol. 2001;166:4826-4830.
43. Griffith JD, Comeau L, Rosenfield S, et al. Mammalian telomeres end in a large duplex loop. Cell. 1999;97:503-514.
44. Bailey SM, Comforth MN, Kurimasa A, Chen DJ, Goodwin EH. Strand-specific postreplicative processing of mammalian telomeres. Science. 2001; 293:2462-2465.
45. Weng NP, Palmer LD, Levine BL, Lane HC, June CH, Hodes RJ. Tales of tails: regulation of telomere length and telomerase activity during lymphocyte development, differentiation, activation, and aging. Immunol Rev. 1997;160:43-54.
46. Weng N, Levine BL, June CH, Hodes RJ. Regulation of telomerase RNA template expression in human T lymphocyte development and activation. J Immunol. 1997;158:3215-3220.
47. Wu KJ, Grandori C, Amacker M, et al. Direct activation of TERT transcription by c-MYC. Nat Genet. 1999;21:220-224.
48. Greenberg RA, O'Hagan RC, Deng H, et al. Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. Oncogene. 1999;18: 1219-1226.
49. Oh S, Song YH, Kim UJ, Yim J, Kim TK. In vivo and in vitro analyses of Myc for differential promoter activities of the human telomerase (hTERT) gene in normal and tumor cells. Biochem Biophys Res Commun. 1999;263:361-365.
50. Gamble DA, Schwab R, Weksler ME, Szabo P. Decreased steady state c-myc mRNA in activated T cell cultures from old humans is caused by a smaller proportion of T cells that transcribe the c-myc gene. J Immunol. 1990;144:3569-3573.
51. Poole JC, Andrews LG, Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene. 2001;269:1-12.
52. Vulliamy T, Marrone A, Goldman F, et al. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. Nature. 2001;413:432-435.

IMMUNOBIOLOGY

THE IMMUNE SYSTEM IN HEALTH AND DISEASE

Charles A. Janeway, Jr.
Yale University Medical School

Paul Travers
Birkbeck College, London University

CB CURRENT BIOLOGY LIMITED

Current Biology Ltd
London, San Francisco and Philadelphia

Blackwell Scientific Publications
OXFORD

GP

Garland Publishing Inc
New York and London

8-19 Fc receptors activate natural killer cells to destroy antibody-coated targets.

Surveillance of cell surfaces for foreign peptides signaling the presence of intracellular infections is largely the province of cellular immune responses mediated by effector T cells. However, antibodies can be produced against viral proteins expressed on the surface of infected cells, and cells bound by such antibodies can be killed by a specialized non-T, non-B lymphoid cell called a **natural killer (NK) cell**. Natural killer cells are large lymphoid cells with prominent cellular granules that make up a small fraction of peripheral blood lymphoid cells. These cells bear no known antigen-specific receptors but are able to recognize and kill a limited range of abnormal cells. They were first discovered because of their ability to kill some tumor cells, but are now known to play an important part in innate immunity, as will be discussed in Chapter 9.

The destruction of antibody-coated target cells by natural killer cells is called **antibody-dependent cell-mediated cytotoxicity (ADCC)** and is triggered when antibody bound to the surface of a cell interacts with Fc receptors on the natural killer cell (Fig. 8.30). NK cells express the Fc receptor FcγRIII (CD16). FcγRIII recognizes the IgG1 and IgG3 subclasses and triggers cytotoxic attack by the NK cell on antibody-coated target cells by mechanisms exactly analogous to those we have encountered in cytotoxic T cells, involving the release of cytoplasmic granules containing perforin and granzymes. The importance of ADCC in defense against infection with bacteria or viruses has not yet been fully established. However, ADCC does represent yet another mechanism by which an effector cell lacking specificity for antigen could mediate antigen-specific functions by utilizing the recognitive capacity of antibody molecules engaged through an Fc receptor.

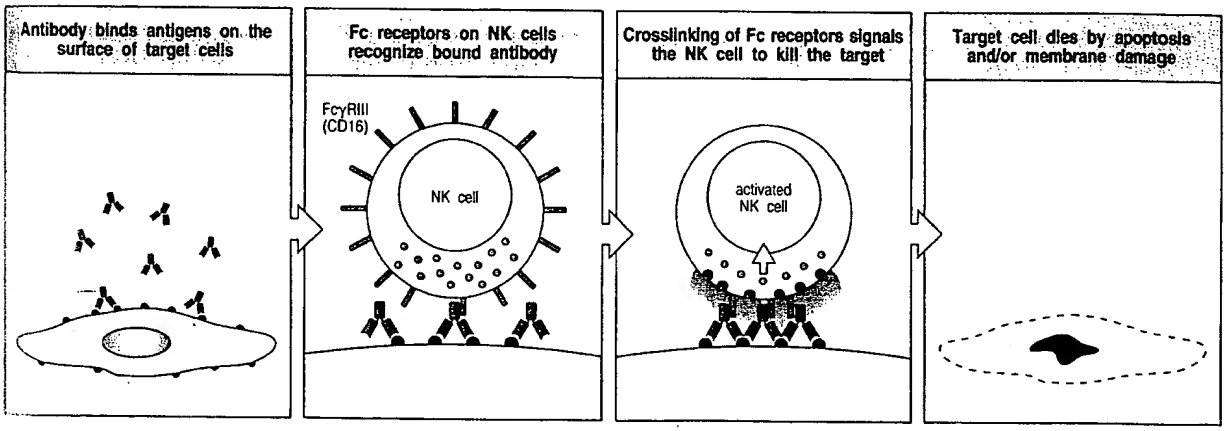

Fig. 8.30 Antibody-coated target cells can be killed by natural killer (NK) cells in antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells are large granular non-T, non-B cells that have FcγRIII (CD16) receptors. When these cells encounter cells coated with IgG antibody, they rapidly kill the target cell. The importance of ADCC in host defense or tissue damage is still controversial.

Immortalization of Human CD8$^+$ T Cell Clones by Ectopic Expression of Telomerase Reverse Transcriptase[1]

Erik Hooijberg,[2]* Janneke J. Ruizendaal,[2]* Peter J. F. Snijders,† Esther W. M. Kueter,* Jan M. M. Walboomers,† and Hergen Spits[3]*

Replicative senescence of T cells is correlated with erosion of telomere ends. Telomerase plays a key role in maintaining telomere length. Therefore, it is thought that telomerase regulates the life span of T cells. To test this hypothesis, we have over-expressed human telomerase reverse transcriptase in human CD8$^+$ T cells. Ectopic expression of human telomerase reverse transcriptase led to immortalization of these T cells, without altering the phenotype and without loss of specificity or functionality. As the T cells remained dependent on cytokines and Ag stimulation for their in vitro expansion, we conclude that immortalization was achieved without malignant transformation. *The Journal of Immunology,* **2000, 165: 4239–4245.**

The ends of linear eukaryotic chromosomes, which are called telomeres, consist of DNA-protein complexes ending in a large duplex loop (1). They serve to maintain chromosomal integrity and prevent end-to-end fusion of the chromosomes. Telomere length is not constant over time. The telomeric ends have a length of 5–15 kb in humans and shorten by 50–100 bp per cell division in normal somatic cells (2, 3). When telomeric ends get too short, cells will enter a state of replicative senescence followed by crisis and cell death. Thus, telomere shortening may prevent unlimited proliferation of human somatic tissues. Telomere shortening is counteracted by the ribonucleoprotein enzyme complex called telomerase, which has two key components, the telomerase reverse transcriptase (TERT)[4] and telomerase RNA, which is used as a template to elongate telomeric ends (for reviews see Refs. 4–6). The crucial role of human (h)TERT in maintaining telomeric length and subsequently of the replicative life span of cells has been demonstrated recently. It has been documented that ectopic expression of hTERT, in cell types without endogenous expression of hTERT, led to elongation of the telomeres and to an increased life span of foreskin fibroblasts, retinal pigment epithelial cells, and endothelial cells (7–9), indicating that hTERT by itself regulates the life span of these cell types. In other cases, however, ectopic expression of hTERT was not sufficient and had to be accompanied by an inactivated Rb/p16^{INK4a} pathway to give similar effects in human mammary epithelial cells and foreskin keratinocytes (10)

A correlation between telomere shortening and life span has also been found in cells of the immune system. It was reported that the average telomeric length and the replicative potential are higher in naive T cells as compared with memory T cells from the same donor, in both CD4$^+$ cells (11, 12) and CD8$^+$ cells (13, 14). These findings are consistent with two ex vivo studies with peripheral blood leukocytes, indicating a correlation between the loss of telomere repeat fragments and the age of the donor (15, 16). Ongoing shortening of telomeres and subsequent induction of replicative senescence in cells of the immune system occur despite the presence of endogenous hTERT in T and B cells (17, 18). The levels of telomerase activity in peripheral blood T and B lymphocytes are regulated at the level of hTERT transcription (19–22), but post-transcriptional mechanisms may also play an important role in the control of the function of the enzyme (23). Activation of T cells by strong stimuli like PMA and ionomycin (20), but also milder stimulation by a combination of CD3 and CD28 Abs (19, 24) or by the cognate Ag presented by the appropriate target cell (25), can induce a transient expression of telomerase. Recently, it was shown that telomerase is up-regulated and telomere length is preserved after virus-induced clonal expansion of CD8$^+$ T cells (26). Despite the endogenous expression and activation induced up-regulation of hTERT in subsets of human T cells, presumably resulting in maintenance of replicative potential in vivo (18), Ag-specific T cell clones cannot be expanded in vitro beyond 20–25 population doublings (PD; reviewed in Ref. 17). This finding raises the question whether the replicative life span of T cells is solely regulated by hTERT. This issue is investigated in the present study.

We examined the effect of ectopic expression of hTERT on the life span of two CD8$^+$ CTL clones, derived from a melanoma patient, which are specific for the Mart-1/Melan-A$_{27-35}$, the Tyrosinase$_{368-376}$ epitopes, respectively, and restricted by HLA-A2. Ectopic expression of hTERT led to a dramatic extension of the life span of these T cell clones, without altering the phenotype, the specificity, and the function of the cells. T cells ectopically expressing hTERT remained dependent on cytokines and Ag stimulations for their in vitro expansion. These findings indicate that constitutive expression of hTERT is necessary and sufficient to extend the life span of CD8$^+$ memory T cells.

*Department of Immunology, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, and †Department of Pathology, Section Molecular Pathology, University Hospital Free University, Amsterdam, The Netherlands

Received for publication April 14, 2000. Accepted for publication July 24, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant 96-1275 from the Dutch Cancer Society, Amsterdam.

[2] Current address: Department of Pathology, Section Molecular Pathology, University Hospital Free University, De Boelelaan 1117, NL-1081HV, Amsterdam, The Netherlands. E-mail address: erik.hooijberg@azvu.nl

[3] Address correspondence and reprint requests to Dr. Hergen Spits, Department of Immunology, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, NL-1066CX, Amsterdam, The Netherlands. E-mail address: hergen@nki.nl

[4] Abbreviations used in this paper: TERT, telomerase reverse transcriptase; hTERT, human TERT; GFP, green fluorescent protein; PD, population doubling; TRAP, telomeric repeat amplification protocol.

0022-1767/00/$02.00

Materials and Methods

Cell lines

The virus-producing cell line Phoenix, the melanoma cell line Mel-AKR, and the EBV-transformed B cell lines JY and EBV-AKR, were grown in medium consisting of Iscove's medium (Life Technologies B.V., Breda, The Netherlands) supplemented with 5–10% FCS (BioWhittaker, Verviers, Belgium), penicillin, and streptomycin (Boehringer Mannheim, Mannheim, Germany).

T cell blasts and T cell clones

T cell blasts were prepared by incubation of 5×10^5 PBMC per ml in Yssel's medium supplemented with 1% normal human serum and 2 μg/ml PHA. T cell blasts and Ag-specific T cell clones were cultured as previously described (27). Briefly, 3×10^5 cells/well were stimulated weekly with a mixture of 1×10^6 irradiated (80 Gy instead of the previously used 30–50 Gy) allogeneic PBMC/ml and 1×10^5 irradiated EBV-B cells (JY), supplemented with 100 ng/ml PHA and 20 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) in Yssel's medium. In some experiments, T cell clones were cultured with cytokines without weekly stimulations. The following concentrations of cytokines were used: 20 IU/ml IL-2, 10 ng/ml IL-7 (PeproTech, Rocky Hill, NJ), and 10 ng/ml IL-15 (Peprotech). Cell cultures were kept in incubators at 37°C in humidified air containing 5% CO_2.

Construction of the retroviral hTERT vector

The full-length coding sequence of hTERT was isolated from pGRN145 (kindly provided by Geron, Menlo Park, CA) as a 3.5-kb *Eco*RI-*Not*I fragment. The hTERT fragment was subsequently ligated into the polylinker of LZRS-linker-internal ribosomal entry site-GFP (28). Correct cloning of hTERT was confirmed by restriction enzyme analysis. This construct designated LZRS-hTERT-IRES-GFP was used to produce retroviral supernatant as previously described (28, 29). As control, we used LZRS-polylinker-IRES-GFP.

Transduction method

The recombinant human fibronectin fragments transduction procedure (RetroNectin; Takara, Otsu, Japan) was based on a method originally developed by Hanenberg et al. (30) with the modifications described by Heemskerk et al. (28). T cells were prestimulated with PHA (31) or with a feeder cell mixture containing PHA and IL-2 for 32–48 h before transduction. Subsequently, the target cells were plated on RetroNectin-coated dishes (maximum 5×10^6 cells/petri dish with a diameter of 3 cm) in 0.5 ml of complete medium mixed with 1 ml of thawed retroviral supernatant. Cells were cultured at 37°C for 6 h or overnight, washed, and transferred to 24-well culture plates (Falcon plastics; Becton Dickinson Labware, Mountain View, CA). The capability of hTERT retrovirus to induce telomerase activity was determined in cultures of primary human keratinocytes devoid of detectable telomerase activity (data not shown).

Flow cytometric analysis

CD2, CD3, CD4, CD8, CD25, CD27, CD28, CD38, CD45, CD45RA, CD45RO, CD54, CD69, CD80, anti-TCR$\alpha\beta$, anti-HLA-DR, and anti-HLA-class I mAbs (all from Becton Dickinson) directly labeled with PE were used for flow cytometric analysis. HLA-A2 tetramers containing the Mart-1/Melan-A_{27-35}, the Tyrosinase$_{368-376}$, or the influenza-A_{58-66} epitope labeled with PE were prepared as previously described (28). Stained cells were analyzed using a FACScan (Becton Dickinson), and the data were processed with CellQuest computer software.

Chromium release assays

Cytotoxicity of T cell clones was determined using a standard chromium release assay previously described (32). All assays were performed in the presence of a 50-fold excess of unlabeled K562 cells to block nonspecific lysis of the target cells. The spontaneous release varied between 10 and 25% of the maximum. SD of triplicate determinations never exceeded 10% of the mean.

Measurement of telomerase activity and hTERT mRNA levels

Cell lysates for analysis of telomerase activity were prepared from $\sim 1 \times 10^6$ cells using the CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) detergent lysis method as described before (33, 34). Briefly, cells were suspended in 50 μl lysis buffer. After homogenization, the suspension was placed on ice for 30 min. Subsequently, the cells were spun down at maximum speed in a microcentrifuge for 30 min at 4°C. The supernatant was transferred to a new tube and snap-frozen in liquid nitrogen. The amount of protein was determined using the Bio-Rad protein detection kit (Bio-Rad Laboratories, Veenendaal, The Netherlands). From the resulting pellet, RNA was isolated using RNAzolB (Campro Scientific, Veenendaal, The Netherlands) for subsequent hTERT mRNA analysis.

Telomerase activity was determined in samples containing various amounts of protein (representative for 100–10,000 cells used) by the telomeric repeat amplification protocol (TRAP). The TRAPeze kit (Oncor, Gaithersburg, MD) was used according to instructions of the manufacturer. To determine the specificity of the assay, the protein samples were preheated for 10 min at 70°C to inactivate telomerase and tested in parallel experiments. No stepladder patterns were observed after this preheating step. Following separation of TRAP products on polyacrylamide gels, autoradiography was performed overnight at −80°C using intensifying screens. Relative telomerase activities were quantified by densitometric evaluation of the TRAP ladder bands relative to the corresponding internal controls. Semiquantitative RT-PCR for hTERT was performed essentially as previously described (34) except that 25 instead of 30 PCR cycles were run to ensure linearity of the amplification reaction. First-strand cDNA synthesis was performed on 100 ng of total RNA using antisense primers for both hTERT and the housekeeping gene snRNP U1A in a single reaction. Subsequent RT-PCR for hTERT and snRNP U1A were performed in a single reaction as well, using primers previously described (34). Resulting PCR products were run in duplicates on the same agarose gel and blotted to the same nylon membrane, followed by hybridization with radiolabeled hTERT and snRNP U1A-specific oligonucleotide probes, respectively. Signal intensities were measured after exposure of the hybridized filters to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Relative levels of hTERT mRNA were calculated according to the following formula: intensity ratio hTERT/snRNP U1A.

Telomere length assay

For measurement of telomere length, genomic DNA was extracted from $\sim 1.5 \times 10^6$ cells using the PureGene DNA extraction kit (Gentra Systems, Minneapolis, MN). Five micrograms of DNA were digested with *Bgl*II (Boehringer Mannheim) and electrophoresed through a 0.7% agarose gel before Southern blotting. The blot was subsequently hybridized to a [γ-^{32}P]ATP end-labeled telomeric oligoprobe $(TTAGGG)_4$. Autoradiography was performed for 5 days. To assess the median telomere length, the peak signal intensity was determined by PhosphorImager analysis at a shorter exposure time to avoid problems of overexposure of the signal.

Results

Enhancement of expansion of human T cells upon ectopic expression of hTERT

We introduced hTERT cDNA in a human $CD8^+$ T cell clone to test the effect of ectopic expression of hTERT on expansion and extension of the life span of human T cells. The T cell clone (AKR-IL7-clone no. 4) has been obtained after stimulation of patient-derived peripheral T cells with autologous melanoma cells genetically engineered to produce IL-7 (E. Hooijberg, J. J. Ruizendaal, and H. Spits, manuscript in preparation). This T cell clone is specific for the HLA-A2-restricted Mart-1/Melan-A_{27-35} epitope. After establishment of this T cell clone, the cells were cultured for 6–8 wk before transduction. In this period, the cells went through 2 PD per week, giving a total number of 12–16 PD.

To address the question whether the introduction of hTERT had an effect on T cell expansion, we followed the expression of green fluorescent protein (GFP) upon further culturing of hTERT-IRES-GFP and control-GFP-transduced T cells. The level of GFP expression in hTERT-IRES-GFP-transduced cells showed an exponential increase over time in two independent experiments with T cells from the same clone from different frozen batches (Fig. 1). In a period of 7 wk, the percentage of hTERT-IRES-GFP-positive cells increased from the initial 5% and 3%, respectively, measured 5 days after retroviral transduction, to 95% on day 53 (Fig. 1). The percentage of hTERT-IRES-GFP-positive cells remained stable (~95%) for another period of 5 wk, after which this experiment of coculturing hTERT-IRES-GFP-positive and -negative cells was terminated. Based on weekly counting of the cells and the percentages of GFP-positive and -negative cells, we calculated the

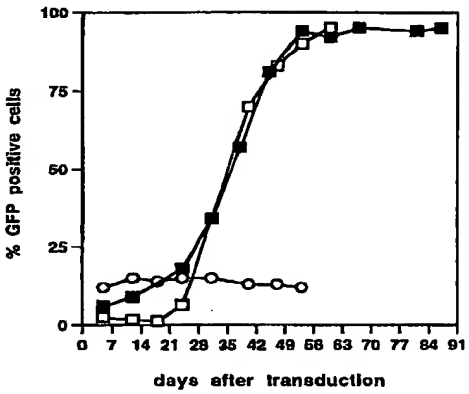

FIGURE 1. Boosted expansion of hTERT-transduced human T cells. T cells were transduced with a retrovirus containing hTERT-IRES-GFP or the marker gene, GFP, only. The T cell clone used here is a tumor-specific, $CD8^+$, HLA-A2-restricted clone recognizing the Mart1/MelanA$_{27-35}$ epitope. Indicated is the percentage of cells positive for the marker in T cells transduced with hTERT-IRES-GFP (■) or the control GFP only (○) over time (given in days). The cultures were stimulated with a feeder cell mixture every week. On day 32, the cells were sorted in a GFP-positive and a GFP-negative fraction for further studies. The result of a second hTERT-IRES-GFP transduction experiment with a different batch of T cells of the same clone is also indicated (□).

average weekly expansion of hTERT-IRES-GFP-positive T cells and of the untransduced cells to be 10-fold (3 PD) and 5-fold (2 PD), respectively.

The higher expansion rate of hTERT-IRES-GFP transduced T cell fraction was not due to the retroviral-mediated gene transfer procedure or to the integration of the provirus into the genome of the T cell clone. Transduction with GFP-only control virus led to expression of the marker in 12% of the T cells as measured 5 days after the transduction (Fig. 1). The level of GFP expression remained stable in this T cell culture for a period of 53 days, after which the cells went into crisis and died. Thus, the maximum number of PD of the control-transduced cells was 30–35, which is comparable to that of the untransduced cells of this clone (data not shown). These data indicate that retroviral transduction per se does not lead to enhanced expansion or extension of the life span of T cells.

Extension of the life span of human T cells upon ectopic expression of hTERT

The experiments shown in Fig. 1 strongly suggest that ectopic expression of hTERT results not only in an increased expansion but also in an extension of the life span of the cells. To obtain more information on the degree of life span extension of the hTERT-transduced cells, we performed subcloning experiments. The maximum number of PD of established $CD8^+$ human T cell clones is in the order of magnitude of 20–30 (for a review see Ref. 17), consistent with experiences in our lab. As mentioned above, the Mart-1/Melan-A$_{27-35}$-specific T cells went through 12–16 PD as an established T cell clone before they were transduced with hTERT. On day 32 after the transduction, we sorted part of the hTERT-transduced cells into a GFP-positive and a GFP-negative fraction. These cells were cloned by single cell disposition using a FACStar^{Plus}. We observed growing cells in the wells seeded with cells from the GFP-negative fraction (12/480) and the GFP-positive fraction (61/480). The clones derived from the positive fraction were, as expected, all GFP-positive, and the clones from the negative fraction were all negative for GFP (data not shown). All subclones, either GFP-negative or GFP-positive, were shown to be positive for staining with HLA-A2 tetramers containing the Mart-1/Melan-A$_{27-35}$ epitope, indicating that all subclones obtained were indeed derived from the same parental clone (data not shown). A second single cell cloning was performed with two randomly selected subclones from the GFP-positive pool and the two best growing clones from the GFP-negative pool. This cloning experiment only yielded subclones from the hTERT-IRES-GFP-positive T cell fraction (10/386 and 5/386). Three clones were isolated for further studies: A11, D8, and F6 (all subclones from subclone no. 1 of clone AKR-IL7 no. 4). The finding that the hTERT transduced no. 4 $CD8^+$ T cell clone could be successfully subcloned twice confirms the notion that ectopic expression of telomerase leads to a dramatic extension of the life span.

Ectopic expression of hTERT in human T cells does not alter functional and phenotypic characteristics

After having established that ectopic expression of hTERT leads to an extended life span, we examined whether it affected the cell surface phenotypes, the specificities, and the functions of the transduced T cells. No differences between the original T cell clone and the hTERT-transduced subsubclones were found in the expression of any of the cell surface markers tested (Table I). It is of note that the original clone as well as the hTERT-transduced T cells were negative for CD27 and CD28, and positive for CD45RO, which is the typical phenotype of memory T cells (35).

To examine whether ectopic expression of hTERT affected the specificity of the T cells, we tested these four clones (wild type no. 4, A11, D8, and F6) for binding of HLA-A2-Mart-1/Melan-A$_{27-35}$ tetramers. Not only the original clone but also the three stained with the HLA-A2-Mart-1/Melan-A$_{27-35}$ tetramers, but not with control tetramers (Fig. 2*A*). Consistent with this observation, the parental clone and the three hTERT transduced subsubclones lysed autologous melanoma cells, and peptide (Mart-1/Melan-A$_{27-35}$) loaded EBV-B cells to the same extent (Fig. 2*B*). As expected, none of the clones lysed untreated EBV-B cells.

Table I. *Phenotypic analysis of wild-type AKR-IL7 no. 4 and hTERT-transduced subsubclones A11, D8, and F6*[a]

Antigen	Expression Levels	
	Wild-Type AKR-IL7 no. 4	Sub-Sub-Clones
IgG control	–	–
TCR αβ	+	+
CD3	++	++
CD4	–	–
CD8	++	++
CD25	±	±
CD27	–	–
CD28	–	–
CD38	++	++
CD45	++	++
CD45RA	±	±
CD45RO	+	+
CD54	+	+
CD69	+	+
CD80	+	+
HLA-class I	+	+
HLA-DR	++	++

[a] The original clone and three isolated hTERT-transduced subsubclones, which, at the time of analyses, differed >45 PD in life span, were phenotyped. T cells were stained with PE-labeled antibodies and analyzed on a FACScan. Gates were set to contain live cells only. Indicated are the levels of expression in four categories as follows: MFI < 10, –; MFI 10–50, ±; MFI 50–500, +; MFI > 500, ++.

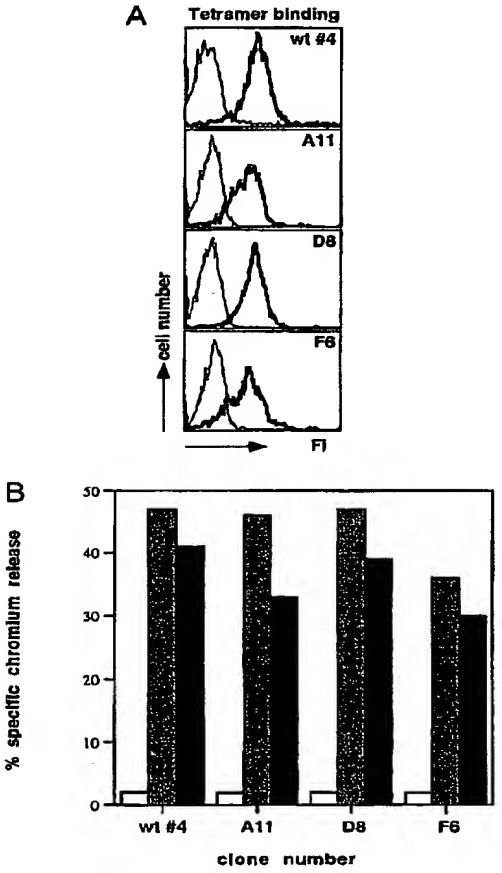

FIGURE 2. Functional analyses of wild-type and hTERT-transduced T cells. *A*, Cells from the wild-type AKR-IL7-no. 4 T cell clone (*wt #4*) and of three subsubclones (*A11*, *D8*, and *F6*) were stained with tetramers containing the HLA-A2-restricted Mart1/MelanA$_{27-35}$ epitope (thick lines). As a negative control, tetramers containing the HLA-A2-restricted influenza epitope were used (thin lines). *B*, These T cells were also used in a cytotoxicity assay on autologous EBV-B cells (□), peptide-loaded EBV-B cells (▨), and on the autologous melanoma cells (■). Indicated is the percentage of specific chromium release of triplicate determinations. The E:T ratio was 60. The data shown are representative for three independent experiments.

hTERT-transduced T cells are still dependent on cytokines and Ag stimulation for survival and expansion

An extension of the life span of somatic cells may lead to abnormal growth characteristics. We examined the growth characteristics of the three isolated subsubclones (A11, D8, and F6) in the absence and presence of cytokines. We cultured the hTERT-transduced T cells in medium without cytokines, or with added IL-2, IL-7, IL-15, or mixtures of these cytokines. IL-7 alone was not capable of supporting survival of either of the subsubclones, nor of the original clone. The hTERT-transduced cells could be maintained in medium containing IL-2 or IL-15 for a period up to 3 mo (longer periods were not tested) without significant expansion. Like the original, untransduced clone, the subsubclones remained highly dependent on periodic activation for their growth. The expansion of T cell cultures in a feeder cell mixture containing IL-2 and PHA was about 5 for the original clone (2 PD per week) and about 10 for the subsubclones (3 PD/wk). These findings demonstrate that T cells expressing ectopic hTERT maintained normal growth characteristics for prolonged periods of time.

Increased hTERT mRNA levels, telomerase activity, and stabilization of telomeres in T cells ectopically expressing hTERT

We next examined the hTERT mRNA levels, telomerase activity, and telomere lengths in untransduced and hTERT-transduced T cells. Ten days after sorting GFP-negative and -positive cells, the levels of hTERT mRNA and of telomerase activity were assayed in the sorted cells. Semiquantitative RT-PCR for measurement of hTERT mRNA levels, relative to a household gene, was performed on day 42 after retroviral transduction of the wild-type T cell clone no.4, 1 wk after the last stimulation with a feeder cell mixture. At that time point, the feeder cells were completely cleared. The hTERT mRNA levels were about 5-fold increased in GFP-positive cells compared with the GFP-negative counterparts (Fig. 3*A*). Telomerase activity was high in both cell fractions and could be detected in as little as 5 ng of protein lysate, which is equivalent to ~100 cells (Fig. 3*B*). Densitometric scanning of TRAP ladder bands compared with the internal control yielded a two to three times higher OD in the lanes corresponding to the GFP-positive cells, indicating a 2- to 3-fold increased telomerase activity. Moreover, a slight decrease in mean telomere length (median length of 6–6.5 kb vs 7 kb) and a smear indicating loss of telomere integrity was observed in the GFP-negative cells compared with the GFP-positive T cell fraction (Fig. 3*C*). At the time of analysis, 12 PD had occurred in the GFP-negative and 18 in the GFP-positive cell populations since the transduction. A larger difference in telomere length between wild-type and the hTERT-transduced cells may be expected near the point of senescence of the wild-type clone no. 4. Unfortunately, this could not be verified because it is difficult to obtain enough material from the GFP-negative fraction or of wild-type T cells close to the point of senescence. Accurate measurement of hTERT mRNA levels, telomerase activity, and telomere lengths is also severely hampered by the presence of contaminating feeder cells, in such near-senescent cultures. In cultures with well-growing T cell clones, these irradiated feeder cells are rapidly cleared. To obtain independent proof for the biological activity of the transduced hTERT in the T cell clones, we compared the telomere lengths of untransduced wild-type T cells of the same clone with the isolated subsubclones A11, D8, and F6. Fig. 3*D* shows that stabilization of telomere length had occurred in the hTERT-IRES-GFP-positive T cell clones, which were subcloned twice (A11, D8, and F6). After being subcloned twice, these clones were maintained in in vitro culture for another 11 wk, which involves on average ~3 PD per week. At the time of telomere length analysis, these cells differed 60–70 PD from the cells analyzed 10 days after sorting and of the wild-type cells used in this analysis. It is apparent that the telomeres in these clones (A11, D8, F6) had not eroded and were maintained at an average length of 7 kb (Fig. 3*D*).

Rescue of a tyrosinase-specific T cell clone by ectopic expression of telomerase

To demonstrate that immortalization by ectopic expression of hTERT is not restricted to clone no. 4, we transduced another T cell clone with hTERT-IRES-GFP. This HLA-A2-restricted, CD8$^+$, T cell clone (AKR-IL7-clone no. 108) is specific for the Tyrosinase$_{368-376}$ epitope, and has been obtained from the same stimulation experiment as AKR-IL7-clone no. 4. Clone no. 108 was selected from our collection of tumor-specific T cell clones because it has been very difficult to grow and expand, with a weekly PD of less than one. Two days after the transduction, the

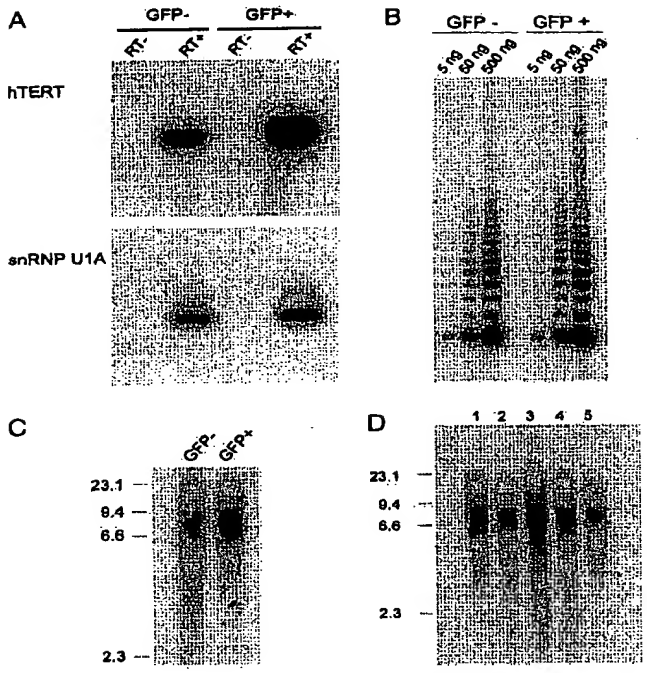

FIGURE 3. Determination of mRNA levels, telomerase activity, and telomere length in wild-type and hTERT-transduced T cell clones. T cell cultures were stimulated every week with a feeder cell mixture. One week after stimulation, hTERT mRNA levels, telomerase activity, and telomere lengths were determined. Experimental details are given in *Materials and Methods*. *A*, The levels of mRNA of hTERT and the household gene snRNP U1A were compared between GFP-positive and GFP-negative cell fractions 10 days after cell sorting, which was 7 wk after transduction. RT^- and RT^+ indicate cDNA synthesis in the absence or presence of added reverse transcriptase respectively. The relative hTERT mRNA levels were about 5-fold higher in GFP-positive cells compared with the GFP-negative counterparts. *B*, Telomerase activity was measured down to 5 ng of protein, which is equivalent to 100 cells, derived from cell lysates of the same cell fractions mentioned above. IC indicates the internal control. Densitometric scanning of TRAP ladder bands compared with the internal control yielded a two to three times higher OD in the lanes corresponding to the GFP-positive cells, indicating a 2- to 3-fold increased telomerase activity. *C*, Telomere lengths were determined on GFP-positive and GFP-negative cells 10 days after sorting, which is 7 wk after retroviral transduction of the cells. At the time of analysis, 12 PD had occurred in the GFP-negative fraction and 18 in the GFP-positive fraction since retroviral transduction. *D*, Telomere lengths were also determined on early passage wild-type AKR-IL7 no. 4 T cells (*lane 1*), and on three subsubclones, A11, D8, and F6, in *lanes 2*, *3*, and *4* respectively. These clones differed 60–70 PD from the GFP-positive and -negative fractions used in *C*. *Lane 5*, A 10-PD later passage of subsubclone D8. A kb marker is indicated on the left of *C* and *D*.

GFP expression, as an indirect measure for telomerase expression, was very low (1–2%; Fig. 4). The low transduction efficiency was most likely due to the low level of proliferation of this clone. After 4 wk, we observed a significantly higher percentage of GFP-positive cells in this culture. Following kinetics similar to those seen with clone no. 4, we observed an increase to 97% 10 wk after transduction. The transduced cells went through 2 PD a week. Subclones of hTERT-transduced no. 108 have been obtained as well. The *inset* in Fig. 4 shows staining with control and Tyrosinase$_{368-376}$ tetramers, demonstrating that the hTERT-transduced cells have retained their specificity. Cytotoxicity assays confirmed

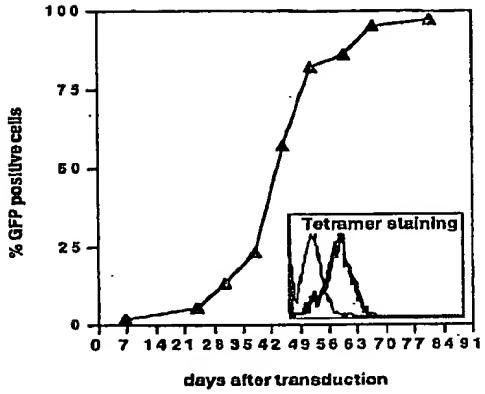

FIGURE 4. A tyrosinase-specific T cell clone is rescued from a total loss by ectopic expression of hTERT. An HLA-A2-restricted, Tyrosinase$_{368-376}$-specific, $CD8^+$, T cell clone (#108) was transduced with hTERT-IRES-GFP retrovirus. Indicated is the percentage GFP-positive cells over time (given in days). The inset shows staining of hTERT-transduced T cells with specific Tyrosinase$_{368-376}$ tetramers (thick lines) and with nonspecific influenza-A tetramers (thin lines). These stainings were performed 10 wk after transduction of the cells.

functionality of these cells as autologous melanoma cells, and peptide-loaded EBV-B cells were lysed, whereas EBV-B cells without exogenous peptide were not (data not shown).

Discussion

T cells express hTERT upon activation (23). Despite the presence of telomerase, the telomere ends of T cells cultured in vitro erode, giving rise to replicative senescence (20, 36). This raises the question whether the replicative life span of T cells is regulated solely by hTERT. This is a relevant question because some cell types like keratinocytes require interference with cell cycle control mechanisms as well as ectopic hTERT expression for significant extension of their replicative life span (10). Here, we investigated the life span of two $CD8^+$ T cell clones transduced with hTERT. The subsubclones of the hTERT-transduced no. 4 T cells have undergone >100 PD at the time of writing, which is much more than the maximum number of PD of the untransduced cells or of control-transduced no. 4 clone (35 PD) and of other T cell clones reported previously (17, 37). These data indicate that constitutive expression of hTERT dramatically extends the life span of this $CD8^+$ CTL clone to a point that one can speak of immortalization. Additional deliberate interference with the cell cycle was not necessary to confer life-span extension to these T cells. The immortalization should be caused by constitutive hTERT expression because transduction of T cells with the GFP control virus did not lead to an increased expansion, nor to an extension of their life span. The constitutive hTERT expression in hTERT-transduced no. 4 cells correlated with a 5-fold higher hTERT mRNA expression and 3-fold higher enzymatic activity compared with untransduced T cells as measured 7 days after the last stimulation. Expansion of no. 4 T cells requires restimulation of these cells every 7–10 days. The cells do not become "resting" in this period of time. To determine whether hTERT transduction into $CD8^+$ T cells results in sustained mRNA expression and enzymatic activity independent of the activation stage of the cells, we used freshly isolated $CD8^+$ T cells. These bulk T cells can be maintained alive without repeated restimulation for a more prolonged period of time than the cloned T cells. T cells transduced with hTERT-IRES-GFP

or with GFP-control virus were isolated and restimulated with PHA and feeder cells. The hTERT-transduced $CD8^+$ T cells showed high levels of hTERT mRNA expression and enzymatic activity on days 7, 10, 14, and 22 after stimulation. The levels of expression and activity were comparable at all time points. In contrast, both mRNA expression and enzymatic activity had dropped to baseline levels already 7 days after activation of GFP-control transduced $CD8^+$ T cells and of untransduced control T cells (results not shown). Because after 2–3 wk these three bulk $CD8^+$ T cell fractions have become in a resting state, we conclude that hTERT transduction leads to sustained mRNA expression and telomerase activity independent of the activation stage of the T cells. The notion that hTERT transduction results in a sustained functional expression is strongly supported by the observation that the subclones A11, D8, and F6 had the same telomere length of 7 kb as the wild-type clone at the time of transduction, despite the fact that the subsubclones underwent 60–70 PD after the transduction. Based on the average loss of telomere length of 50–100 bp per PD in T cells (2, 3, 20), one would expect to observe a minimum telomere end erosion of 3–3.5 kb in cell samples differing 60–70 PD. The maintenance of the telomere lengths in the $hTERT^+$ subsub clones proves the biological activity of the transduced hTERT and indicates a correlation between the observed increase in life span of the T cells and constitutive activity of the transduced hTERT. However, whether hTERT expression is the sole cause of immortalization is not yet sure. One may argue that immortalization of the T cells is the result of hTERT expression combined with another event induced by the retroviral transduction itself either in all hTERT-transduced cells or in a very small proportion of the transduced cells. We cannot exclude these possibilities, although we consider it unlikely that a rare event that occurred in a minority of the cells which together with telomerase is responsible for the observed immortalization. We observed that, on the average, the transduced T cells underwent 3 PD and the untransduced cells 2 PD per week. Assuming that all transduced cells had a growth advantage compared with untransduced cells, one can calculate the percentage of $hTERT\text{-}GFP^+$ cells expected after 6 wk of culture to increase from 5% to 76%. We observed an increase from 5% to 60% in one and from 3% to 60% in the second experiment (Fig. 1). These observations indicate that the majority of the hTERT- transduced cells have a growth advantage compared with wild-type cells. Recently, we have transduced a panel of $CD4^+$ T cell clones and observed also with these clones an expansion advantage of all hTERT-transduced cells in comparison with untransduced cells (H. Yssel and H. Spits, unpublished observations). Another question is whether all hTERT-transduced cells enter a state of immortalization. The low cloning efficiency (15%) of $hTERT\text{-}GFP^+$ no. 4 cells indicates that not all transduced cells are immortalized and may suggest that the site of integration of the hTERT DNA is a determining factor for immortalization. However, the cloning efficiencies of the no. 4 subclones, which should have hTERT integrated at a single site in the genome, were even lower (1.5% and 3% in two experiments with different subclones). A likely explanation for the low cloning efficiency of these subclones is that a proportion of the transduced cells lose their hTERT over time either by inactivation of the inserted DNA or by posttranscriptional inactivation.

Recently, it was reported by Liu et al. (23) that low levels of hTERT mRNA are constitutively present in $CD4^+$ T lymphocytes, regardless of the telomerase activity in the cells that was activation-induced and transient, suggesting that telomerase is mainly regulated at a posttranscriptional level. However, it is possible that only a minority of the $CD4^+$ T cells in the study of Liu et al. expressed constitutive telomerase mRNA levels. Moreover, while our data do not exclude posttranscriptional regulation of hTERT activity, they strongly suggest that the level of hTERT transcripts is the main regulator of the life span of T cells. Presently, we analyze the effect of constitutive hTERT expression on a large panel of $CD8^+$ and $CD4^+$ T cell clones to obtain insight into the regulation of telomerase in T cells in general.

It is of note that the stabilization of the telomere length as observed in the subsubclones of clone no. 4 indicates that constitutive hTERT expression does not result in telomere lengthening as has been observed in hTERT-transfected fibroblasts (7, 8) and in germinal center B cells (18, 38). The reasons for this have yet to be determined. The finding that ectopic expression of telomerase extends the replicative life span of certain cell types without net telomere lengthening is not unprecedented. In human fibroblasts transfected with hTERT, the telomeres continue to shorten to average lengths below those of untransfected cells that enter replicative senescence and crisis (39, 40). This suggests that hTERT has a role in life-span extension beyond the prevention of telomere length erosion.

Importantly, ectopic expression of hTERT did not affect the phenotype, Ag specificity, or functionality of the T cells. Of equal importance is the observation that the hTERT-transduced T cells are still dependent on cytokines and Ag stimulation for proliferation, suggesting that these T cells were not transformed. These findings are well in agreement with recent publications on the lack of induction of a transformed phenotype in hTERT-transfected normal human fibroblasts and retinal pigment epithelial cells (41, 42). The advantages of immortalized T cell clones are obvious. The availability of immortalized functional T cells with defined specificities will facilitate functional, genetic, and biochemical experiments. The limited in vitro life span of human Ag-specific T cell clones has severely hampered application of these cells in cellular therapies involving adoptive transfer of Ag-specific T cells. Our finding that tumor-specific CTL clones can be immortalized by ectopic expression of hTERT and thus can be expanded to very large numbers may lead to potential application of these cells in the treatment of cancer patients.

Acknowledgments

We thank the Geron Corporation for plasmid pGRN145 containing hTERT cDNA, Drs. L. Ma and R. D. Steenbergen for excellent technical assistance and helpful discussions, and E. Noteboom and A. Pfauth for cell sorting.

References

1. Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, and T. de Lange. 1999. Mammalian telomeres end in a large duplex loop. *Cell 97:503.*
2. Allsopp, R. C., H. Vaziri, C. Patterson, S. Goldstein, E. V. Younglai, A. B. Futcher, C. W. Greider, and C. B. Harley. 1992. Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. USA 89:10114.*
3. Harley, C. B., A. B. Futcher, and C. W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature 345:458.*
4. Blackburn, E. H. 1992. Telomerases. *Annu. Rev. Biochem. 61:113.*
5. Lustig, A. J. 1999. Crisis intervention: the role of telomerase. *Proc. Natl. Acad. Sci. USA 96:3339.*
6. Hodes, R. J. 1999. Telomere length, aging, and somatic cell turnover. *J. Exp. Med. 190:153.*
7. Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright. 1998. Extension of life-span by introduction of telomerase into normal human cells. *Science 279:349.*
8. Vaziri, H., and S. Benchimol. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol. 8:279.*
9. Yang, J., E. Chang, A. M. Cherry, C. D. Bangs, Y. Oei, A. Bodnar, A. Bronstein, C. P. Chiu, and G. S. Herron. 1999. Human endothelial cell life extension by telomerase expression. *J. Biol. Chem. 274:26141.*
10. Kiyono, T., S. A. Foster, J. I. Koop, J. K. McDougall, D. A. Galloway, and A. J. Klingelhutz. 1998. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature 396:84.*

11. Weng, N. P., B. L. Levine, C. H. June, and R. J. Hodes. 1995. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proc. Natl. Acad. Sci. USA 92:11091.*
12. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708.*
13. Monteiro, J., F. Batliwalla, H. Ostrer, and P. K. Gregersen. 1996. Shortened telomeres in clonally expanded $CD28^-CD8^+$ T cells imply a replicative history that is distinct from their $CD28^+CD8^+$ counterparts. *J. Immunol. 156:3587.*
14. Hamann, D., S. Kostense, K. C. Wolthers, S. A. Otto, P. A. Baars, F. Miedema, and R. A. van Lier. 1999. Evidence that human $CD8^+CD45RA^+CD27^-$ cells are induced by antigen and evolve through extensive rounds of division. *Int. Immunol. 11:1027.*
15. Frenck, R. W., Jr., E. H. Blackburn, and K. M. Shannon. 1998. The rate of telomere sequence loss in human leukocytes varies with age. *Proc. Natl. Acad. Sci. USA 95:5607.*
16. Rufer, N., T. H. Brummendorf, S. Kolvraa, C. Bischoff, K. Christensen, L. Wadsworth, M. Schulzer, and P. M. Lansdorp. 1999. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J. Exp. Med. 190:157.*
17. Effros, R. B., and G. Pawelec. 1997. Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion? *Immunol. Today 18:450.*
18. Weng, N. P., K. S. Hathcock, and R. J. Hodes. 1998. Regulation of telomere length and telomerase in T and B cells: a mechanism for maintaining replicative potential. *Immunity 9:151.*
19. Buchkovich, K. J., and C. W. Greider. 1996. Telomerase regulation during entry into the cell cycle in normal human T cells. *Mol. Biol. Cell 7:1443.*
20. Bodnar, A. G., N. W. Kim, R. B. Effros, and C. P. Chiu. 1996. Mechanism of telomerase induction during T cell activation. *Exp. Cell Res. 228:58.*
21. Weng, N. P., L. Granger, and R. J. Hodes. 1997. Telomere lengthening and telomerase activation during human B cell differentiation. *Proc. Natl. Acad. Sci. USA 94:10827.*
22. Hiyama, K., Y. Hirai, S. Kyoizumi, M. Akiyama, E. Hiyama, M. A. Piatyszek, J. W. Shay, S. Ishioka, and M. Yamakido. 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol. 155:3711.*
23. Liu, K., M. M. Schoonmaker, B. L. Levine, C. H. June, R. J. Hodes, and N. P. Weng. 1999. Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes. *Proc. Natl. Acad. Sci. USA 96:5147.*
24. Weng, N. P., B. L. Levine, C. H. June, and R. J. Hodes. 1996. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J. Exp. Med. 183:2471.*
25. Hathcock, K. S., N. P. Weng, R. Merica, M. K. Jenkins, and R. Hodes. 1998. Antigen-dependent regulation of telomerase activity in murine T cells. *J. Immunol. 160:5702.*
26. Maini, M. K., M. V. Soares, C. F. Zilch, A. N. Akbar, and P. C. Beverley. 1999. Virus-induced $CD8^+$ T cell clonal expansion is associated with telomerase up-regulation and telomere length preservation: a mechanism for rescue from replicative senescence. *J. Immunol. 162:4521.*
27. Yssel, H., J. E. De Vries, M. Koken, W. van Blitterswijk, and H. Spits. 1984. Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods 72:219.*
28. Heemskerk, M. H., E. Hooijberg, J. J. Ruizendaal, M. M. van der Weide, B. Kueter, A. Q. Bakker, T. N. Schumacher, and H. Spits. 1999. Enrichment of an antigen-specific T cell response by retrovirally transduced human dendritic cells. *Cell. Immunol. 195:10.*
29. Heemskerk, M. H. M., B. Blom, G. Nolan, A. P. A. Stegmann, A. Q. Bakker, K. Weijer, P. C. M. Res, and H. Spits. 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J. Exp. Med. 186:1597.*
30. Hanenberg, H., K. Hashino, H. Konishi, R. A. Hock, I. Kato, and D. A. Williams. 1997. Optimization of fibronectin-assisted retroviral gene transfer into human $CD34^+$ hematopoietic cells. *Hum. Gene Ther. 8:2193.*
31. Dardalhon, V., N. Noraz, K. Pollok, C. Rebouissou, M. Boyer, A. Q. Bakker, H. Spits, and N. Taylor. 1999. Green fluorescent protein as a selectable marker of fibronectin-facilitated retroviral gene transfer in primary human T lymphocytes. *Hum. Gene Ther. 10:5.*
32. Hooijberg, E., P. C. van den Berk, J. J. Sein, J. Wijdenes, A. A. Hart, R. W. de Boer, C. J. Melief, and A. Hekman. 1995. Enhanced antitumor effects of CD20 over CD19 monoclonal antibodies in a nude mouse xenograft model. *Cancer Res. 55:840.*
33. Steenbergen, R. D., J. M. Walboomers, C. J. Meijer, E. M. van der Raaij-Helmer, J. N. Parker, L. T. Chow, T. R. Broker, and P. J. Snijders. 1996. Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. *Oncogene 13:1249.*
34. Snijders, P. J., M. van Duin, J. M. Walboomers, R. D. Steenbergen, E. K. Risse, T. J. Helmerhorst, R. H. Verheijen, and C. J. Meijer. 1998. Telomerase activity exclusively in cervical carcinomas and a subset of cervical intraepithelial neoplasia grade III lesions: strong association with elevated messenger RNA levels of its catalytic subunit and high-risk human papillomavirus DNA. *Cancer Res. 58:3812.*
35. Hamann, D., P. A. Baars, M. H. Rep, B. Hooibrink, S. R. Kerkhof-Garde, M. R. Klein, and R. A. van Lier. 1997. Phenotypic and functional separation of memory and effector human $CD8^+$ T cells. *J. Exp. Med. 186:1407.*
36. Pan, C., B. H. Xue, T. M. Ellis, D. J. Peace, and M. O. Diaz. 1997. Changes in telomerase activity and telomere length during human T lymphocyte senescence. *Exp. Cell Res. 231:346.*
37. Pawelec, G., A. Rehbein, K. Haehnel, A. Merl, and M. Adibzadeh. 1997. Human T-cell clones in long-term culture as a model of immunosenescence. *Immunol. Rev. 160:31.*
38. Weng, N., B. L. Levine, C. H. June, and R. J. Hodes. 1997. Regulation of telomerase RNA template expression in human T lymphocyte development and activation. *J. Immunol. 158:3215.*
39. Zhu, J., H. Wang, J. M. Bishop, and E. H. Blackburn. 1999. Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Proc. Natl. Acad. Sci. USA 96:3723.*
40. Prescott, J. C., and E. H. Blackburn. 1999. Telomerase: Dr. Jekyll or Mr. Hyde? *Curr. Opin. Genet. Dev. 9:368.*
41. Jiang, X. R., G. Jimenez, E. Chang, M. Frolkis, B. Kusler, M. Sage, M. Beeche, A. G. Bodnar, G. M. Wahl, T. D. Tlsty, and C. P. Chiu. 1999. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat. Genet. 21:111.*
42. Morales, C. P., S. E. Holt, M. Ouellette, K. J. Kaur, Y. Yan, K. S. Wilson, M. A. White, W. E. Wright, and J. W. Shay. 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat. Genet. 21:115.*